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THE MINERAL EXCHANGES OF MAN

II. EFFECT OF EXCESS POTASSIUM AND OF CALCIUM ON TWO NORMAL MEN AND ON AN OEDEMATOUS NEPHRITIC*

By

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THE manner in which the body marshals its defenses against the excessive ingestion of inorganic acid radicals has been demonstrated by the results of several investigations (15, 35, 19, 14). There is less unanimity of opinion however in regard to the mechanism of the action of such cations as potassium and calcium.

In 1873 Bunge (11) attempted to explain the desire of herbivorous animals for common salt on the basis of excessive amounts of potassium in their diets. He showed that the diet of the ox contained from three to nine equivalents of potassium for each equivalent of sodium, whereas the diet of a carnivorous animal such as the cat contained not more than two equivalents of potassium for each equivalent of sodium. In the case of herbivora, he assumed that this ingestion of large amounts of potassium tended to raise the potassium content of the body fluids above the normal level with the result that the excess potassium was rapidly excreted by the kidney. He further postulated that during the period of transportation of potassium by the blood there was some exchange of anions between sodium and potassium salts. This exchange of anions led to an increase of sodium phosphate, sodium bicarbonate, etc., in the body fluids so that these substances were also present in amounts exceeding their renal threshold, hence sodium was excreted in the urine. Because of the considerable amount of sodium chloride normally present in blood, the anions

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derived from sodium salts by potassium were chiefly chlorine ions and these were excreted in combination with potassium. The end result was an excessive excretion of both sodium and chlorine.

As proof of this hypothesis he cited several experiments performed upon himself. After following the excretion of sodium, potassium and chlorine in his urine for several days, during which he lived on a carefully controlled diet, he took a very large dose of dipotassium phosphate (equivalent to 12.7 gm. of K) and noted a marked increase in the excretion of sodium, potassium and chlorine in the urine over a period of 24 hours. On the following day, when no potassium salt was added to his diet, the urinary sodium and chlorine excretion was distinctly less than during the control period. In a subsequent experiment an equivalent dose of potassium citrate produced identical effects upon the excretion of sodium and chlorine.

Meyer and Cohn (22) in 1911 noted that infants increased in weight when 0.4 to 0.7 per cent of the total weight of their diets consisted of sodium chloride or disodium phosphate. Weight loss occurred when the sodium salt was replaced by potassium or calcium salts. Several balance experiments were carried out during two-day periods. The intake and output of calcium, sodium, potassium, phosphorus and chlorine was observed on a constant diet, and the effect on the balance determined, following addition of sodium chloride, potassium bicarbonate, and calcium chloride and acetate to this diet. Of these salts sodium chloride had the least effect on the balance of the other cations and anions studied, while potassium bicarbonate produced an unfavorable effect on the balance of all but potassium. Calcium chloride which may be regarded as an acid-forming salt (17, 14) produced a distinctly unfavorable effect on the balances of both sodium and potassium.

The experiments of Bunge and Meyer and Cohn were of too short duration to determine whether this mobilization and excretion of minerals could be long enough continued to bring about any serious depletion of the mineral stores of the body.

Miller (23) followed the excretion of sodium and chlorine in the urine of pigs fed on a diet consisting chiefly of starch and noted that the addition of 11 to 14 grams of potassium acetate or phosphate to the daily diet produced an immediate increase in the excretion of sodium and chlorine in the urine. The effect was only temporary, and after a day or two of continued high potassium feeding the urinary excretion of sodium fell to a level even below that of the control period.

Following this (1926) he repeated these experiments on rats (24) and

determined the total excretion of calcium, phosphorus, sodium and chlorine, while the rats were on a constant diet, and again after the addition of a potassium salt to the basal ration. Observations were continued over an interval of about 2 weeks. The immediate effect was an excessive excretion of sodium and chlorine with a prompt return nearly to the basal level. There also appeared to be a slight increase in the excretion of both calcium and phosphorus while the animals were on the high potassium diet. Although a balance was not kept, Miller concluded that the increase in excretion of minerals during the high potassium feedings was too small to have a deleterious effect upon the mineral reserve of the animals.

Richards, Godden and Husband (27) (1924) observed the effect of adding large amounts of sodium chloride to the diets of growing pigs. An immediate increase in the potassium content of the urine was noted which was almost completely balanced by diminished fecal excretion of potassium. The effect on nitrogen, calcium and phosphorus retention was favorable. In later experiments (1927) (27) when potassium citrate was substituted for sodium chloride in the pig's diet, there was decreased assimilation of nitrogen, phosphorus and calcium. In respect to sodium the authors state in conclusion

> The results obtained for growing pigs with regard to sodium excretion do not support the theory of sodium impoverishment put forward by Bunge as the result of experiments on himself.

Of the various potassium salts employed by different investigators the bicarbonate, acetate, citrate, phosphate and to a lesser degree, the chloride, have been found to produce immediate unfavorable effects upon the sodium balance, suggesting that the action of these salts is in some measure dependent upon the cation.

Opinion as to the mechanism of the action of calcium salts does not appear to be as uniform. Blum, Aubel and Hausknecht (7) maintain that the diuresis which sometimes follows the administration of calcium salts, is the result of direct antagonism between calcium and sodium and does not necessarily depend upon the nature of the anion. They cite an experiment performed upon an oedematous subject in whom diuresis was produced by alternate ingestion of calcium chloride and calcium lactate. At first small doses of these salts were effective but as the oedema diminished, increasingly large doses of salt became necessary to produce diuresis. The elimination of sodium, chlorine and potassium was followed in the urine. No correlation could be found between the excretion of potassium or chlorine and weight loss. On the other hand retention of sodium was

followed by increase in weight while excretion of sodium was associated with weight loss.

Haldane, Hill and Luck (17) assume that the diuretic action of calcium chloride is the result of an acidosis and Gambel, Ross and Tisdall (14) have shown that the acidosis is caused by the greater absorption of the chlorine ion than of the calcium ion. This is in accord with the work of Gamble, Blackfan and Hamilton (13) who believe the mechanism of the action of ammonium chloride and calcium chloride to be similar, both producing an acidosis. The ammonia-forming mechanism of the kidney (25) and the increase in acidity of the urine are not sufficient to compensate entirely for the hydrochloric acid formed from chlorine ingested, hence base is withdrawn from the blood and tissues, chiefly sodium and potassium. Results in agreement with these have been reported by Linder (19) following the administration of hydrochloric acid to normal men and to patients with nephritis.

In order to observe the effect of potassium upon the balances of the other cations in man, we have fed potassium citrate to two normal subjects and to a patient with nephritis and oedema. By way of comparison a known acid-forming salt—calcium chloride[1]—was then administered to the same subjects.

Experimental

The experiment was divided into three periods of 5 or 6 days[2] each, during which the balances of nitrogen, sodium, potassium, calcium, magnesium, iron, phosphorus and chlorine were determined.

The total dietary intake per period was computed from the mineral analysis of standard diets prepared in the metabolism kitchen. The excretion of nitrogen and minerals was determined from analyses of samples of urine and stool. (Methods of analysis, management of the metabolism units, etc., have been reported in a previous publication (4).)

During a preliminary or control period, only the standard diets were eaten by the subjects of the experiment. In the following periods a known

[1] In the following experiments it is to be regretted that more of the variable factors were not eliminated at the time the experiments were in progress. Subsequent analysis of the collected data has shown that accurate interpretation of the results obtained during the period of administration of calcium chloride was impossible owing to the variation in dosage of the calcium salt, the simultaneous administration of parathormone and lack of adequate control periods between the high potassium and calcium periods. The criticisms in regard to control periods and dosage are also applicable, but to a lesser degree, in the period in which potassium citrate was administered. Since it is difficult to separate completely that portion of the experiment which may be significant from that which is unimportant, and since it is hoped that our experience may prevent others from encountering similar pitfalls, the entire data have been presented.

[2] Results given in tables and charts have been computed on the basis of 5-day periods.

amount of potassium citrate or calcium chloride was taken in addition to the diet. The salts given were in solution and were administered in divided doses during the course of the day. Only the total amount of salt ingested during the period is recorded in the tables and charts. In each case the daily dose is approximately 1/5 of the total for the period.

Dietary and other factors which may influence the mineral metabolism have been summarized by Bauer, Albright and Aub (5). Briefly these are:

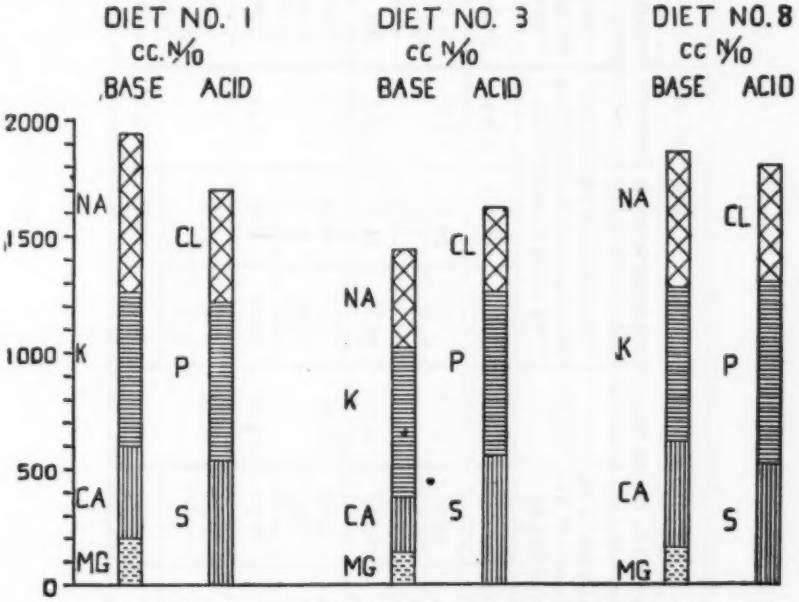

CHART 1. Acid and basic components of ash of three typical diets expressed in cc. of N/10 acid and base.

a.—level of mineral intake; b.—acid base values of diet; c.—relative amounts of presumably antagonistic cations in the diet; d.—accessory food factors—particularly vitamin D; e.—factors relative to age, weight, sex and activity of the subject.

A detailed description of the standard diets has been given elsewhere (4). It is believed that these diets were sufficient in respect to calories, protein, accessory food factors, and mineral content to maintain a normal healthy adult in mineral balance. In Chart 1 the acid base values of three typical examples of the ten diets used have been recorded graphically. The various acid and basic components have been expressed in terms of cubic

TABLE I

NITROGEN AND MINERAL BALANCES IN TWO NORMAL SUBJECTS AND AN OEDEMATOUS NEPHRITIC. CONTROL PERIOD 5 DAYS

Element	Normal Subject Z Age 24 yrs. Wt. at beginning of period 57.4 kg. Wt. at end of period 56.8 kg.					Normal Subject S Age 23 yrs. Wt. at beginning of period 73.9 kg. Wt. at end of period 74.6 kg.			Subject M Nephritis Age 44 Wt. at beginning of period 75.8 kg. Wt. at end of period 75.9 kg.		
	Intake		Output		Balance	Intake	Output	Balance	Intake	Output	Balance
Nitrogen	Food	—	Urine	75.0		—	76.7		—	41.3	
		78.7	Stool	6.1		93.2	4.6		52.6	4.15	
	Total	78.7		81.1	−2.4	93.2	81.3	+11.9	52.6	45.45	+7.15
Phosphorus	Food	—	Urine	4.55		—	5.78		—	3.97	
		8.64	Stool	3.62		10.07	3.14		5.96	2.42	
	Total	8.64		8.17	+0.47	10.07	8.92	+1.15	5.96	6.39	−0.43
Chlorine	Food	—	Urine	17.1		—	12.88		—	2.93	
		11.93	Stool	—		13.66			8.42	—	
	Total	11.93		17.1	−5.17	13.66	12.88	+ 0.78	8.42	2.93	+5.49

Sodium	Food	— 10.29	Urine Stool	10.58 0.12		— 11.16	7.66 0.29		— 6.80	2.02 0.15	
	Total	10.29		10.70	−0.41	11.16	7.95	+ 3.21	6.80	2.17	+4.63
Potassium	Food	— 18.97	Urine Stool	13.70 2.25		— 21.76	13.40 3.11		— 13.00	12.68 1.27	
	Total	18.97		15.95	+3.02	21.76	16.51	+5.25	13.00	13.95	−0.95
Calcium	Food	— 5.19	Urine Stool	0.88 4.40		— 5.76	1.12 3.99		— 4.04	0.03 4.44	
	Total	5.19		5.28	−0.09	5.76	5.11	+0.65	4.04	4.47	−0.43
Magnesium	Food	— 1.62	Urine Stool	0.32 1.12		— 1.80	0.69 0.86		— 1.05	0.39 0.71	
	Total	1.62		1.44	+0.18	1.80	1.55	+0.25	1.05	1.10	−0.05
Iron	Food	— 0.098	Urine Stool	0.002 0.080		— 0.109	0.005 0.069		— 0.064	0.003 0.370	
	Total	0.098		0.082	+0.016	0.109	0.074	+0.035	0.064	0.373	−0.309

TABLE II

NITROGEN AND MINERAL BALANCES IN TWO NORMAL SUBJECTS AND AN OEDEMATOUS NEPHRITIC. POTASSIUM CITRATE ADMINISTRATION*——5 Day Period

Element	Normal Subject Z Age 24 yrs. Wt. at beginning of period 57.1 kg. Wt. at end of period 57.9 kg. Extra K as Potassium Citrate K = 14.22 gm in 5 days 7.86 gm. Potassium Citrate per diem.					Normal Subject S Age 23 yrs. Wt. at beginning of period 74.7 kg. Wt. at end of period 74.8 kg. K as Potassium Citrate K = 19.33 gms. in 5 days 10.69 gm. Potassium Citrate per diem			Subject M Nephritis Age 44 yr. Wt. at beginning of period 75.5 kg. Wt. at end of period 73.9 kg. K as Potassium Citrate K = 37.05 gm. in 5 days 20.5 gm. Potassium Citrate per diem		
	Intake		Output		Balance	Intake	Output	Balance	Intake	Output	Balance
Nitrogen	Food	— 110.7	Urine 95.0 Stool 7.7			— 91.80	73.90 6.20		— 53.60	45.50 2.84	
	Total	110.7		102.7	+8.0	91.80	80.10	+11.7	53.60	48.34	+5.26
Phosphorus	Food	— 11.85	Urine 5.50 Stool 5.47			— 9.98	5.34 4.01		— 5.68	2.51 1.64	
	Total	11.85		10.97	+0.88	9.98	9.35	+0.63	5.68	4.15	+1.53
Chlorine	Food	— 16.44	Urine 14.85 Stool —			— 13.72	13.25		— 7.82	24.66	
	Total	16.44		14.85	+1.59	13.72	13.25	+0.47	7.82	24.66	−16.84

Sodium	Food	— 13.82	Urine Stool	8.79 0.51		— 11.25	7.49 0.10		— 5.85	13.25 0.54	
	Total	13.82		9.30	+4.52	11.25	7.59	+3.66	5.85	13.79	−7.94
Potassium	Medication Food	14.22 26.66	Urine Stool	31.10 4.19		19.34 22.15	34.20 2.90		37.05 12.49	38.40 1.37	
	Total	40.88		35.29	+5.59	41.49	37.10	+4.39	49.54	39.77	+9.77
Calcium	Food	— 7.20	Urine Stool	1.01 6.53		— 6.30	0.93 5.16		— 3.44	0.16 3.14	
	Total	7.20		7.54	−0.34	6.30	6.09	+0.21	3.44	3.30	+0.14
Magnesium	Food	— 2.25	Urine Stool	0.73 1.52		— 1.81	0.67 1.11		— 0.98	0.19 0.50	
	Total	2.25		2.25	0.00	1.81	1.78	+0.03	0.98	0.69	+0.29
Iron	Food	— 0.143	Urine Stool	0.002 0.109		— 0.118	0.002 0.084		— 0.057	0.007 0.036	
	Total	0.143		0.111	+0.032	0.118	0.086	+0.032	0.057	0.043	+0.014

* In the normal subjects this period followed immediately after control period, in the nephritic subject it was preceded by $CaCl_2$ period.

TABLE III

NITROGEN AND MINERAL BALANCES IN TWO NORMAL SUBJECTS AND AN OEDEMATOUS NEPHRITIC. CALCIUM CHLORIDE ADMINISTRATION*
Normal Subjects–6 Day Period Calculated to 5 Day Period; Nephritic Subject–5 Day Period

Element	Normal Subject Z Age 24 yrs. Wt. at beginning of period 57.9 kg. Wt. at end of period 57.0 kg. Ca in $CaCl_2$=17.8 gm. Cl_2 in $CaCl_2$=31.5 gm. 9.86 gm. $CaCl_2$ per diem Parathormone 100 Units on 5th Day of Period					Normal Subject S Age 23 yrs. Wt. at beginning of period 75.2 kg. Wt. at end of period 73.4 kg. Ca in $CaCl_2$=23.5 gm. Cl_2 in $CaCl_2$=41.70 gm. 13.04 gm. $CaCl_2$ per diem Parathormone 100 Units on 5th Day of Period			Subject M Nephritis Age 44 Yrs. Wt. at beginning of period 76.3 kg. Wt. at end of period 75.9 kg. Ca in $CaCl_2$=4.09 Cl_2 in $CaCl_2$=7.10 2.24 gm. $CaCl_2$ per diem. Parathormone 300 Units 100 Units on 1st, 3rd and 5th day of Period		
	Intake		Output		Balance	Intake	Output	Balance	Intake	Output	Balance
Nitrogen	Food	—	Urine	60.00		—	78.30		—	47.50	
		92.1	Stool	9.75		86.0	6.84		51.9	4.06	
	Total	92.1		69.75	+22.35	86.0	85.14	+0.86	51.9	51.56	+0.34
Phosphorus	Food	—	Urine	4.77		—	6.36		—	4.05	
		10.14	Stool	5.69		9.48	5.22		5.78	2.17	
	Total	10.14		10.46	−0.32	9.48	11.58	−2.10	5.78	6.22	−0.44
Chlorine	Medication	31.5	Urine	35.00		41.70	51.4		7.10	12.97	
	Food	13.77	Stool	—		12.85	—		7.98	—	
	Total	45.27		35.00	+10.27	54.55	51.4	+3.15	15.08	12.97	+2.11

Sodium	Food	—	Urine	10.65		—	9.75		—	3.94	
		10.65	Stool	0.61		9.90	0.46		6.46	0.52	
	Total	10.65		11.26	−0.61	9.90	10.21	−0.31	6.46	4.46	+2.00
Potassium	Food	—	Urine	21.50		—	26.00		—	10.90	
		21.97	Stool	3.93		20.20	3.69		12.89	1.58	
	Total	21.97		25.43	−3.46	20.20	29.69	−9.49	12.89	12.48	+0.41
Calcium	Medication	17.80	Urine	3.21		23.50	3.43		4.09	0.27	
	Food	6.18	Stool	18.80		5.64	23.90		3.70	10.30	
	Total	23.98		22.01	+1.97	29.14	27.33	+1.81	7.79	10.57	−2.78
Magnesium	Food	—	Urine	0.61		—	0.76		—	0.45	
		1.72	Stool	1.29		1.60	1.20		1.03	0.77	
	Total	1.72		1.90	−0.18	1.60	1.96	−0.36	1.03	1.22	−0.19
Iron	Food	—	Urine	0.002		—	0.002		—	0.002	
		0.110	Stool	0.067		0.096	0.085		0.062	0.071	
	Total	0.110		0.069	+0.041	0.096	0.087	+0.009	0.062	0.073	−0.011

* Followed Potassium Citrate period for normal subjects; Control period for nephritic subject.

centimeters of N/10 acid and base. Phosphorus was considered di-basic (33). Inspection of the chart shows that there is no marked preponderance of acid over base or base over acid in the ash of the diets.

Of the three subjects studied, two were medical students aged 24, and 23 years respectively, in good health, who lived in the hospital during the period of observation, and took their meals in the metabolism kitchen. Their activity was in no wise restricted and they were permitted to have as much food and water as desired. In order to meet their energy requirements it was necessary to increase each article on the menu a uniform amount, to provide 50 and 75 per cent, respectively, more energy than was provided for in the original menus of the diets, which were calculated at a 2000 calorie basis.

The third subject was a man aged 44 years, ill with nephritis and oedema. His renal lesion was presumably primarily that of hemorrhagic Bright's disease with later development of secondary degenerative phenomena (1) (see case report). His activity was necessarily considerably restricted although he was not confined to bed but was permitted to be up in a wheel chair as much as he desired. The standard diets averaging about 2000 calories per diem proved adequate in his case.

All three subjects were essentially in nitrogen equilibrium or showed positive nitrogen balances throughout the period of observation.

Discussion of Balances

Summaries of the balances of all elements followed in the three subjects have been recorded in tabular form. Table I shows the balances of the three men during the control period, Table II the balances during the period when potassium citrate was administered, and Table III the balances during the administration of calcium chloride. In order to present some of the data graphically, two charts have been prepared. Chart 2 shows the changes taking place in the metabolism of calcium, magnesium and phosphorus in each subject during the three periods; similarly Chart 3 shows the changes occurring in the metabolism of sodium, potassium and chlorine.

Calcium, Magnesium and Phosphorus Balances. Control Period. An appreciation of the level of intake, mode of excretion, and balances of these elements may best be obtained by reference to Chart 2.

It will be noted that the phosphorus balances are slightly positive in both normal subjects and slightly negative in the nephritic subject during this period. The calcium balance is almost zero in the first normal subject, slightly positive in the second, and slightly negative in the nephritic subject.

The exceedingly low calcium excretion by way of the kidney in Bright's disease, particularly that type in which the tubular lesion predominates,

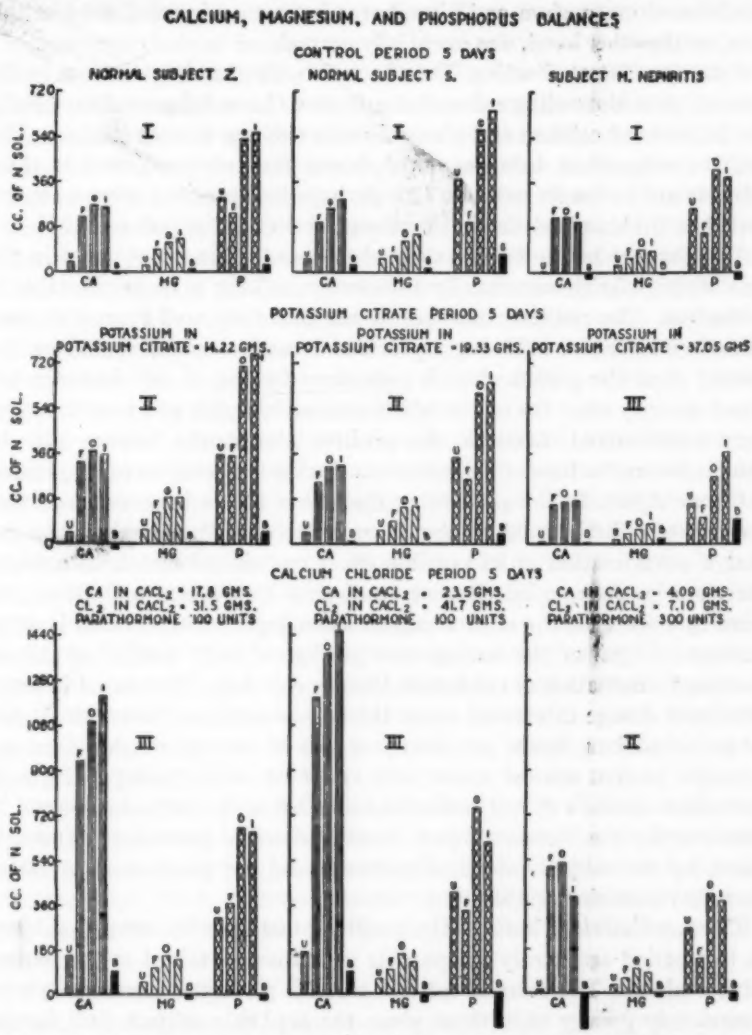
CALCIUM, MAGNESIUM, AND PHOSPHORUS BALANCES
CONTROL PERIOD 5 DAYS
NORMAL SUBJECT Z.
NORMAL SUBJECT S.
SUBJECT M. NEPHRITIS
CC. OF N SOL.
CA
MG
P
POTASSIUM CITRATE PERIOD 5 DAYS
POTASSIUM IN POTASSIUM CITRATE = 14.22 GMS.
POTASSIUM IN POTASSIUM CITRATE = 19.33 GMS.
POTASSIUM IN POTASSIUM CITRATE = 37.05 GMS
CALCIUM CHLORIDE PERIOD 5 DAYS
CA IN CACL2 = 17.8 GMS.
CL2 IN CACL2 = 31.5 GMS.
PARATHORMONE 100 UNITS
CA IN CACL2 = 23.5 GMS.
CL2 IN CACL2 = 41.7 GMS.
PARATHORMONE 100 UNITS
CA IN CACL2 = 4.09 GMS.
CL2 IN CACL2 = 7.10 GMS.
PARATHORMONE 300 UNITS

CHART 2. Columns represent cc. of normal solution per 5 day period. Legend. U = output in urine; F = output in feces; O = total output ($U+F$); I = total intake (food and medication); B = balance, positive above base line, negative below base line. Heavy black line in Ca intake column separates food intake (below) from medication intake (above). Roman numerals indicate chronological order of periods.

has been discussed by Scriver (30), Albright and Bauer (2) and others. In the nephritic patient it amounted to only about 30 mgs. in 5 days. The partition of magnesium and phosphorus between urine and stool in this man, on the other hand, was essentially normal.

Potassium Citrate Feeding. The effect of feeding extra potassium in the form of potassium citrate does not appear to have influenced materially the balances of calcium and phosphorus in the two normal subjects. The positive magnesium balances noted during the previous period in these subjects are no longer present. This perhaps indicates too short a control period on the standard diets rather than an effect due to potassium.

The changes in the balances of calcium and magnesium noted in the man with nephritis were hardly sufficiently striking to be attributable to potassium. The positive balances in his case may well represent compensatory retention following a previous excessive excretion of these elements, since the period of high potassium feeding in this instance followed directly after the one in which calcium chloride and parathormone were administered. Likewise, his positive phosphorus balance may be compensatory for losses of this element during the two preceding periods.

Citrate Effect. In the preceeding discussion it has been assumed that the citrate radical would be almost completely oxidized in the body and that a consideration of its possible effect on mineral metabolism might therefore be disregarded. Recent studies by Proverman and Brull (26) seem to show that the citrate radical when ingested in sufficiently large amounts (1 gm. of the sodium salt per kg. of body weight) causes an increased elimination of calcium in the urine of dogs. Translated in terms of human dosage this would mean the administration of between 60 and 70 gm. of sodium citrate per diem to a man of average weight. Since our nephritic patient showed minor toxic symptoms while taking 20.5 gm. of potassium citrate a day, it is doubtful whether such a large dose would be tolerated by the human subject. Smaller doses of potassium citrate, as taken by the subjects of this experiment, did not produce an increased excretion of calcium in the urine.

Calcium Chloride Feeding. The results obtained in the normal subjects in this period are hardly comparable with those obtained in the patient with nephritis. The doses of calcium chloride given the normal men were enormously greater than those given the nephritic subject. Still further confusion resulted from the fact that parathormone was administered to all three individuals. The normal subjects received but one dose of 100 units of parathormone near the end of this experimental period, while the man with nephritis received a total of 300 units during the course of 5 days.

Calcium. From the observations of Scriver (30) one might be led to discount the effect of parathormone in the particular type of Bright's disease exhibited by the nephritic patient. Two observations made are in accord with those of Scriver: a.—the blood calcium was not raised above the normal level following the administration of parathormone as is usually the case in normal individuals, b.—there appeared to be an excessive fecal calcium excretion leading to a distinctly negative calcium balance. Whether the excessive excretion was due to the effect of parathormone, to lack of absorption of ingested calcium, or to the relative inactivity of the subject, it is impossible to determine. Greenwald and Gross (16) found that the administration of parathyroid extract to dogs for short periods of time increased the fecal excretion of calcium. Albright, *et. al.* (3), on the other hand, concluded that parathormone was without effect on the fecal excretion of calcium in man. This subject, together with observations on the effect of activity on fecal calcium excretion, has been fully reviewed by Bulger, Dixon, and Barr (10).

The urinary calcium excretion was also increased, either due to parathormone or to the administration of an acid-forming calcium salt. Judging from observations made by Albright and Bauer (2) on the effect of ammonium chloride on calcium excretion in "nephritis," the latter factor would appear to be the important one. In the normal subjects, high calcium feeding led to a definite retention of calcium during this period. That this retention may be more apparent than real cannot be denied, since the period of high calcium feeding was not followed by a control period on standard diets. As might be expected, owing to the ingestion of an acid-forming calcium salt, the urinary calcium excretion was greatly increased; nevertheless, the major portion of the ingested calcium appeared in the feces.

Magnesium. The negative magnesium balances in all three subjects are probably due to the acid effect of calcium chloride, since all show a somewhat higher urinary magnesium excretion than during the control period. On the other hand, evidence concerning the effect of parathormone (16, 34) and of the calcium ion (21, 8) on magnesium metabolism is still inconclusive.

Phosphorus. Briggs (9) has called attention to the effect of calcium acetate and calcium chloride on the partition of phosphorus between urine and feces. Feeding large amounts of these salts increased the amount of phosphorus in the feces and diminished it in the urine. The total excretion of phosphorus was diminished. Bulger, Dixon and Barr (10) in discussing phosphorus metabolism in hyperparathyroidism have noted

the essential independence of the absorption of calcium and phosphorus from the intestine in that disease and suggest that their observations are in accord with the studies of Bergeim (6) on the absorption of these two elements. There seems to have been a tendency for the normal subjects in our balance experiments to excrete a somewhat greater proportion of the phosphorus in the stool during the period of high calcium intake than during the control period. The difference in the mode of excretion was not marked however and there was no great preponderance of fecal over urinary excretion in either man. The phosphorus balances were negative. Subject Z, who had a markedly positive nitrogen balance (Table III) during this period, had only a very small negative phosphorus balance, while in Subject S, who was in nitrogen equilibrium, the phosphorus balance was distinctly negative. It is not altogether certain whether these negative phosphorus balances were due to the large doses of calcium chloride (28) ingested or to the effect of parathormone on phosphorus metabolism recently stressed by Albright, *et al.* (3). In summing up the situation three factors must be kept in mind, a.—the effect of the calcium ion tending to increase the excretion of phosphorus in the feces, b.—the effect of parathormone tending to increase urinary phosphorus excretion and c.—the acid effect of calcium chloride which, according to the observations of Fitz *et al.* (12) and Albright and Bauer (2), might also be presumed to cause an increased urinary excretion of phosphorus.

The manner and amount of phosphorus excretion in the man with nephritis varied but little from that of the control period although the balance was negative. It seems probable that neither calcium chloride in the amount given, nor parathormone, influenced the phosphorus metabolism in his case.

Sodium, Potassium and Chlorine Balances

It is at once apparent from a comparison of Charts 2 and 3, that the manner of excretion of these elements differs fundamentally from that of calcium, magnesium and phosphorus. Sodium and potassium, as is well known, are absorbed almost completely from the gastro-intestinal tract and excreted in the urine in combination with various acid radicals. The amount of sodium excreted in the stool is negligible in all our cases; the amount of potassium excreted by this route, while small, is fairly constant and presumably comes from undigested vegetable residue. The chlorine intake is based upon calculations made from tables (29) and the output does not include chlorine lost in the stool.

Control Period. During the control period, one normal subject lost both

sodium and chlorine while the other showed positive balances for both elements. Potassium was retained in each instance. In the case of the patient with nephritis, perhaps due to previous restriction of sodium

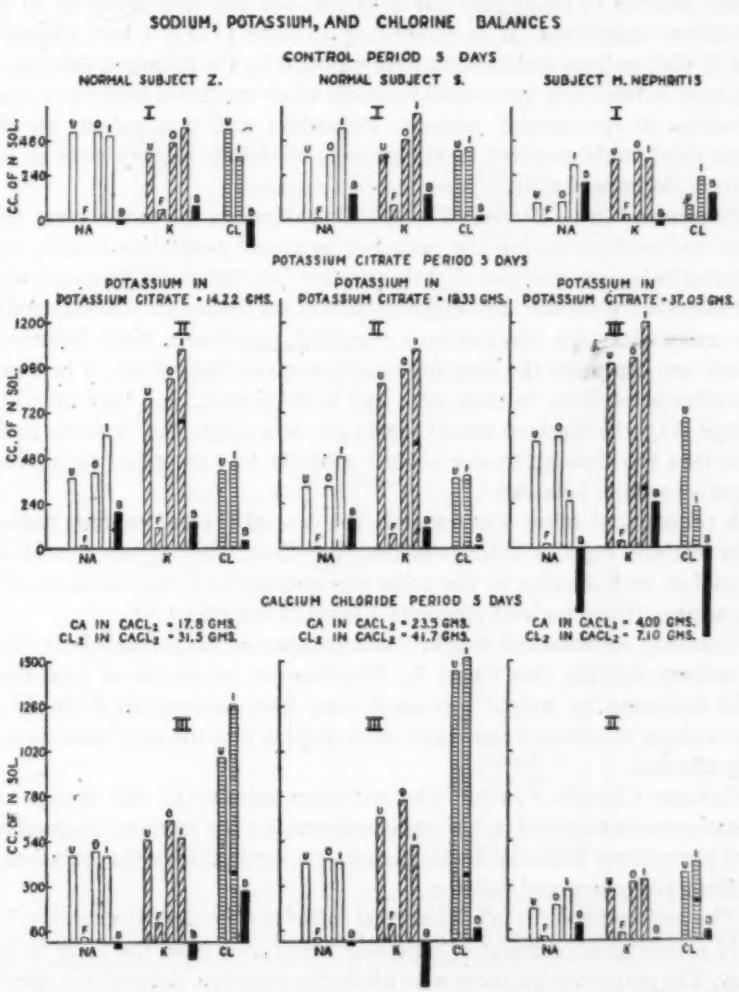
SODIUM, POTASSIUM, AND CHLORINE BALANCES
CONTROL PERIOD 5 DAYS
NORMAL SUBJECT Z.
NORMAL SUBJECT S.
SUBJECT M. NEPHRITIS
CC. OF N SOL.
NA
K
CL
POTASSIUM CITRATE PERIOD 5 DAYS
POTASSIUM IN POTASSIUM CITRATE = 14.22 GMS.
POTASSIUM IN POTASSIUM CITRATE = 18.33 GMS.
POTASSIUM IN POTASSIUM CITRATE = 37.05 GMS.
CALCIUM CHLORIDE PERIOD 5 DAYS
CA IN CACL2 = 17.8 GMS.
CL2 IN CACL2 = 31.5 GMS.
CA IN CACL2 = 23.5 GMS.
CL2 IN CACL2 = 41.7 GMS.
CA IN CACL2 = 4.09 GMS.
CL2 IN CACL2 = 7.10 GMS.

CHART 3. Columns represent cc. of normal solution per 5-day period. Legend. U = output in urine; F = output in feces; O = total output $(U+F)$; I = total intake (Food and medication); B = balance, positive above base line, negative below base line. Heavy black line in K and Cl intake column separates food intake (below) from medication intake (above). Roman numerals indicate chronological order of periods.

chloride or to a tendency on the part of the body to retain the elements of this salt, a large proportion of the ingested sodium and chlorine was retained. A similar retention of potassium in his case did not occur. The actual balance of potassium was negative, but not sufficiently so to be considered significant. It is interesting to note (Table I and Charts 2 and 3) that sodium and calcium were excreted by the damaged kidneys of this man in relatively very small amounts when compared with their renal excretion in the normal subjects. Potassium and magnesium, on the other hand, were excreted in his urine as efficiently as they were in the urine of the normal men.

Potassium Citrate Feeding. The sodium balance in the two normal subjects was positive during the period of increased potassium intake. The chlorine balances were also slightly positive but did not correspond with the retention of sodium. The major portion of the potassium was excreted in the urine although the balances remained positive in both instances. When one compares the dose of potassium given these men—3 to 4 gm. per diem in addition to that contained in their food, with that taken by Bunge (11) who ingested more than 12 gm. in a single day, it seems probable that the dose given our normal subjects was too small to produce negative sodium balances.

A pronounced effect was noted in the case of the oedematous patient who received 7 gm. of extra potassium per diem. The increased excretion of sodium and chlorine in the urine was striking and resulted in marked negative balances for both elements. (Table II and Chart 3.)

Sweating, diuresis and weight loss accompanied the increased excretion of sodium chloride (See Chart 4). The decrease in volume of total body fluid indicated by weight loss must have been accomplished chiefly at the expense of extracellular water since sodium was the only base materially affected.

Calcium Chloride Feeding. The criticisms relative to salt dosage and parathormone administration made in discussing the calcium, magnesium, and phosphorus balances in this period, are applicable to the balances of sodium, potassium and chlorine.

The sodium balances in both normal subjects were slightly negative, but only a very small amount of base was withdrawn from the body in this way. The potassium balances were distinctly negative. Some of the chlorine derived from calcium chloride may have been excreted in combination with potassium, or the negative balances may represent a delay in excretion of potassium, since a control period did not intervene between the periods of high potassium and high calcium intake.

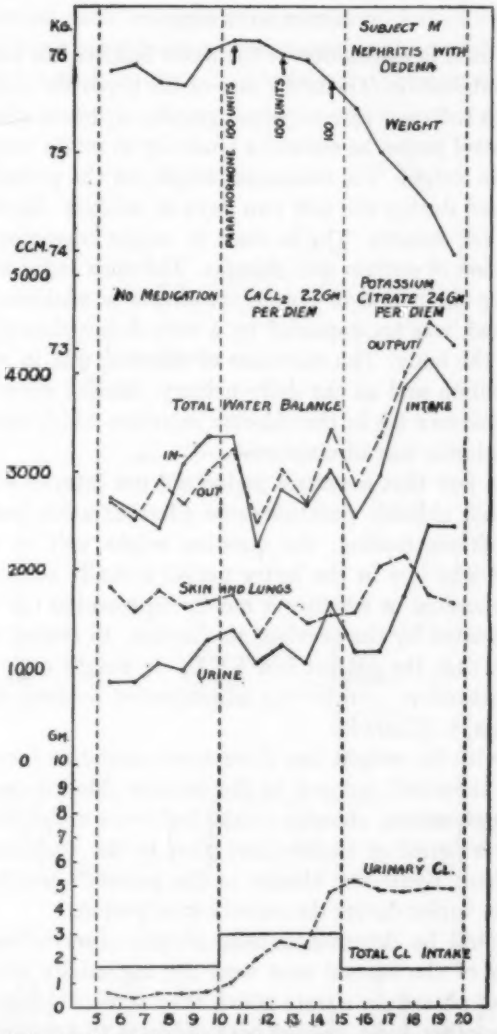

CHART 4. Water balance of Nephritic Subject during mineral metabolism study. Note diuresis, weight loss and increased excretion of chlorine in urine at time potassium citrate was administered.

The chlorine balances were positive throughout but were small except in the case of normal subject Z. The rather large positive chlorine balance in his case may in part have been compensatory for chlorine lost during the control period.

WATER BALANCES

The method used in calculation of the water balance has been described in a previous publication (4). In the case of the nephritic subject this was determined with sufficient care to permit graphic representation (Chart 4). During the control period he showed a tendency to retain water which led to an increase in weight. The maximum weight for the period of observation was attained during the first two days of calcium chloride ingestion and thereafter fell steadily. The increase in weight corresponded roughly with the retention of sodium and chlorine. The most rapid loss of weight occurred during the period of potassium citrate administration, and, as previously stated, was accompanied by a very definite loss of sodium and chlorine from the body. The excretion of chlorine was in excess of the excretion of sodium and as the daily urinary chlorine curve shows, this was probably due to a lag in the chlorine excretion which occurred at the time calcium chloride was administered.

Owing to the fact that a control period did not intervene between the period of calcium chloride parathormone administration and the period of potassium citrate feeding, the question might well be raised as to whether the weight loss in the latter period actually resulted from the ingestion of potassium or whether it merely represented the continuation of a process initiated by the previous medication. In respect to this point it is significant that the patient lost 5.5 kg. in weight on a previous occasion when potassium citrate was administered in doses of 20 gm. per diem for eight days, (Chart 5).

Coincident with the weight loss there was a moderate increase in urine volume and a three-fold increase in the urinary chlorine excretion. Diet, water intake and sodium chloride intake had been maintained at a constant level for a period of twelve days prior to the beginning of the experiment, without significant change in the patient's weight, and these factors were not varied during the experimental period.

So far as could be determined from weight observations alone, the water balances of the normal men were not materially affected by the smaller doses of potassium citrate which they received. Calcium chloride feeding, on the other hand, caused both subjects to lose weight. Subject *Z* who had a strongly positive nitrogen balance during this period, lost 0.9 kg. in weight, while subject S who was in nitrogen equilibrium lost 1.8 kg.

NITROGEN BALANCES

The protein content and caloric value of the diets given the normal subjects approximated the standards given by Atwater (cited by Sherman

(32)) for normal men engaged at moderately active work. Both men showed positive nitrogen balances during part of the period of observation. In the control period, subject Z complained of hunger on a diet whose

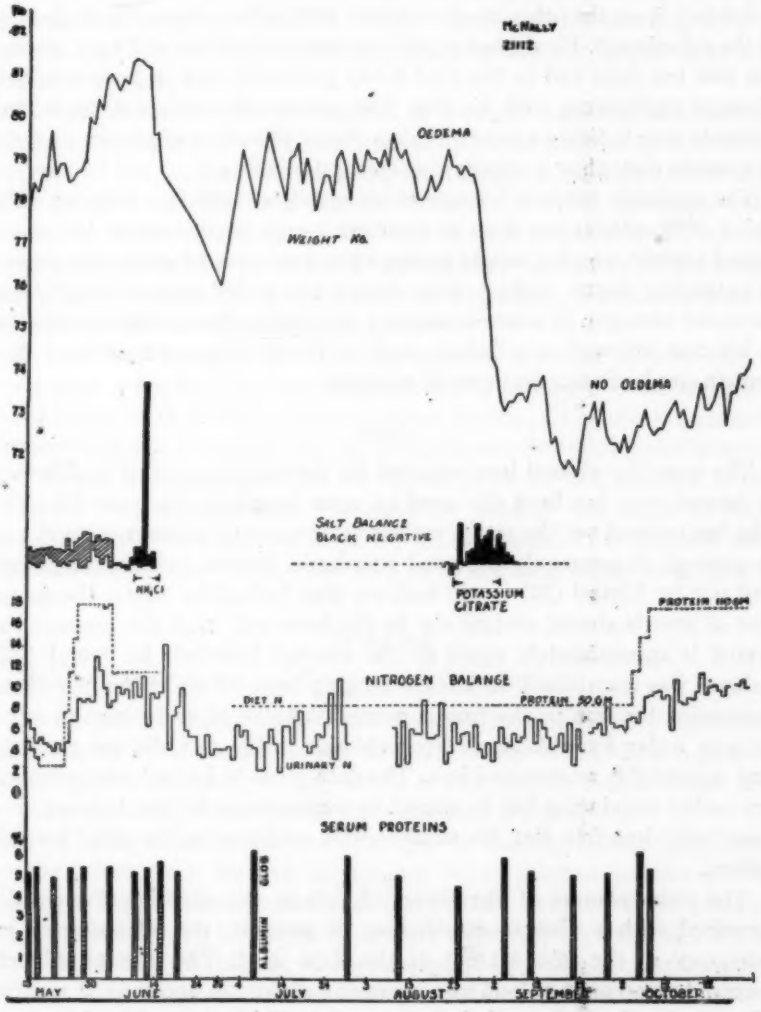

CHART 5. Loss of weight and negative salt balance (sodium chloride as calculated from chlorine excreted in urine) obtained by administration of 20 gm. of potassium citrate per diem to the nephritic subject. Mineral balance not kept at this time.

energy value was about 3000 calories per diem and showed a small negative nitrogen balance. In the two following periods, when the diets had been increased to an energy value of about 3500 calories per diem, he stored approximately 30 gm. of nitrogen.

Subject S, on the other hand, received 3500 calories from the beginning of the experiment. He showed a positive nitrogen balance of 23 gm. during the first ten days and in the final 5-day period seemed to have attained nitrogen equilibrium with his diet. The storage of nitrogen in these two students may indicate a somewhat less liberal allowance of protein in their customary diets than is usually considered desirable.

The nephritic subject, because of his restricted activity, required only about 2000 calories per diem to meet his energy requirements. He maintained a fairly constant weight except when loss occurred during the period of potassium citrate feeding. Even during this period of rapid weight loss he stored nitrogen. It seems reasonable to suppose that nitrogen retention in his case followed as a logical result of the previous restriction of diet protein employed as a therapeutic measure.

Iron

The quantity of food iron required for the maintenance of equilibrium in normal men has been discussed at some length by Sherman (31, 32), who has pointed out the rather serious deficiency of experimental evidence in support of commonly accepted standards. Recent investigations carried out by Lintzel (20) would indicate that in healthy adults the excretion of iron is almost exclusively in the feces and that the amount excreted is approximately equal to the amount ingested. He found that balance was maintained on intakes ranging from 0.9 to 64 mg. per diem. Increasing the iron intake from a normal value of 10 to 13 mgs. to 50 or 60 mgs. a day by addition of ferric chloride to the diet, did not result in any appreciable retention of iron. The data given in Lintzel's experiments are rather convincing but in respect to maintenance of iron balance on a practically iron-free diet, his results await confirmation by other investigators.

The iron balances of the three subjects in our experiment were determined with a view to establishing, if possible, the adequacy or inadequacy of the iron content of the diets used. The normal subjects received an amount of food iron averaging slightly in excess of 21 mg. per diem during the entire period of observation. This was considerably above the standard requirement of 15 mg. per diem suggested by Sherman (32) and probably exceeded the usual daily iron intake of the subjects. Both

men stored iron throughout the experiment. Subject Z retained 87 mg. and subject S, 77 mg., a daily positive balance of over 5 mg. in each case. It is possible that this comparatively large storage of iron in our normal subjects may have been associated with some previous impoverishment of their iron reserves, or perhaps simply represented the building up of a reserve iron supply. The latter supposition is more in accord with experimental evidence (18, 36).

The patient with nephritis received an average daily iron intake from his food of 12.2 mg. During the control period he excreted a very large amount of iron in the stool resulting in a negative balance of 309 mg. Whether this represented a contamination of the stool specimen with iron, or a delayed excretion of medicinal iron given prior to the metabolism study, is not known. That it did not represent a loss of body iron seems fairly certain since in the two remaining periods he remained practically in equilibrium with the iron in his diet.

In only one instance did the daily urinary excretion of iron exceed more than a fraction of a milligram, and this occurred in the nephritic subject during the period of high potassium feeding. Even here the amount excreted (6.7 mg. in 5 days) was too small materially to effect the balance.

It should be emphasized that the figures given for iron excreted in the urine are only approximate. The quantity present was found in most instances to be too small for accurate determination. It is believed that the amount of iron in urine has been overestimated rather than underestimated.

Summary

1. Calcium, Magnesium and Phosphorus Metabolism.

a.—Potassium citrate when added in quantities of 7.8, 10.5 and 24.0 gm. respectively to the diets of two normal men and to the diet of a patient with nephritis and oedema, for periods of five days in each instance, did not alter the metabolism of calcium, magnesium or phosphorus.

b.—The ingestion of 9.8 and 13.0 gm. of calcium chloride per diem by the normal men for five-day periods led to marked increase in the urinary and fecal calcium excretion and at least temporarily to positive calcium balances. In the nephritic subject where the ingestion of 2.2 gm. per diem of calcium chloride was accompanied by intramuscular injection of large doses of parathormone, urinary calcium excretion was increased but the quantity excreted was much less than that excreted by the normal men during the control period. Fecal calcium in his case was excessive and resulted in a distinctly negative calcium balance.

c.—The magnesium balances of all three subjects became negative during the period of calcium chloride—parathormone administration.

d.—The influence of calcium chloride feeding on phosphorus metabolism could not be completely evaluated because of the number of variable factors entering into the experiment.

2. Sodium, Potassium and Chlorine Metabolism.

a.—High potassium feeding for five-day periods did not produce negative balances in the metabolism of sodium and chlorine in the normal subjects.

b.—The much larger doses of potassium citrate taken by the nephritic subject caused a marked increase in the excretion of sodium and chlorine in his urine, which resulted in negative balances for both elements. Diuresis, sweating and weight loss accompanied the increased excretion of sodium and chlorine.

c.—In the normal subjects, sodium and potassium balances were negative during the period of calcium chloride feeding. This is believed to have been due to the acid effect of calcium chloride but the negative potassium balances may have been due to delay in excretion of this element resulting from administration of potassium citrate in the immediately preceding period.

d.—The comparatively small doses of calcium chloride given the man with nephritis did not affect his sodium or potassium metabolism.

3. Attention has been called to the unusually small amount of sodium and calcium excreted in the urine of the nephritic subject during the control period, and this has been contrasted with his normal urinary excretion of potassium and magnesium.

4. The total nitrogen metabolism was not affected by the administration of potassium citrate or calcium chloride.

5. Iron was stored by both normal subjects on an average food iron intake of 21 mg. per diem. In the nephritic subject, if one excludes the control period, a food iron intake of 12 mg. per diem was sufficient to maintain iron balance.

CASE REPORT

SUBJECT M. UNIT NO. 21112

History of Illness: A clerk, aged 44 years, was admitted to the Medical Service of the Strong Memorial Hospital on January 20, 1929, complaining of shortness of breath, palpitation of the heart and swelling of the ankles. His general health had been good until one year prior to admission when he began to suffer from generalized headaches on arising in the morning. These were followed shortly by palpitation of the heart and for the 3 or 4 months immediately preceding admission by shortness of breath. For the past month he had noted considerable swelling of his ankles and had to arise once nightly to urinate.

Past History revealed no evidence of illness or disease which appeared to have any relationship to the condition for which he was admitted.

Family History: Father died age 53 years, supposedly with Bright's disease.

Physical Examination: Temperature 37.C. Pulse 72. Resp. 20. Ht. 169 cm. Wt. 88 kg. The patient was well developed with slight tendency to obesity. There was no orthopnea, dyspnea or cyanosis while at rest. The gums showed moderate pyorrhea and several of the teeth were carious. The pharynx was moderately injected and the tonsils were embedded and reddened. A small amount of pus could be expressed from the crypts of the right tonsil. There were a few moist rales at the lung bases posteriorly. The heart was enlarged to the left, the outer border of cardiac dullness measuring 14 cm. to the left of the mid sternal line in the 5th intercostal space. There was a soft systolic murmur at the apex. Peripheral and retinal arteries showed only a very slight degree of sclerosis. The blood pressure was 160 mm. Hg. systolic and 110 diastolic. The abdomen was soft. The liver and spleen were not palpable. There was moderate pitting oedema over both tibiae.

Laboratory Examinations: Urine—specific gravity 1020. Albumin 4 plus, sugar and acetone negative. Microscopic—a few red blood cells but definitely more than normal, and a few granular casts. Stool examination was normal. Blood—Hb. 100 per cent (Sahli); white blood cells 7,000 with a normal differential leucocyte count; N.P.N. 33 mg. per cent; serum albumin 4.3 per cent globulin 1.6 per cent; blood Wassermann negative. Electrocardiogram showed left ventricular preponderance. X-rays of the chest showed definite cardiac hypertrophy. An intravenous phenolsulphonphthalein test showed 60 per cent excretion of the dye in the urine in two hours. X-rays of the teeth showed some alveoloclasia and pyorrhea.

Hospital Admissions: First Admission, January 20, 1929 to February 17, 1929. The patient remained in bed throughout this period of approximately four weeks. He was placed on a diet of low sodium chloride content with some decrease in the oedema of the lower extremities but this did not entirely disappear. There was a gradual fall in the blood pressure to 132 systolic and 84 diastolic. The rales heard at the lung bases on admission soon cleared and there remained no signs of cardiac decompensation. The urine continued to show a large amount of protein, generally about 6 to 8 gm. per liter but at times as high as 20 gm. per liter. Red blood cells and granular casts were consistently found in the urine. On February 5, 1929 the tonsils were removed, and a small abscess was found in the right tonsil. Although there was no excessive bleeding as the result of the operation, the patient suffered somewhat from shock and recovered slowly. Examination of the blood just prior to discharge showed essentially the same findings as on admission. He was discharged with the advice to rigidly curtail his activity and to have all remaining infected teeth extracted. Dietary restrictions other than keeping sodium chloride intake at a low level were not imposed.

Second Admission: May 14, 1929 to December 21, 1929. The patient was admitted the second time primarily for study of his nitrogen and mineral metabolism. The physical and laboratory examinations were essentially as given for the first admission. During the first two weeks the effect of low and high protein intake on the course of oedema was observed while the patient was on a sodium chloride intake of about 3.5 gms. per diem, and later a low sodium chloride intake (1.5 gms. per diem) was given with a protein intake of about 20 gm. a day. Weight tended to increase during most of the month of observation with perhaps slight increase in the oedema. Blood N.P.N., serum albumin, globulin and total serum protein showed some variation but probably not significant amounts. Average values were N.P.N. 30 mg. per cent, serum albumin 4.5 per cent, serum globulin 0.8 per cent and total serum protein 5.3 per cent. On June 16, 1929 the patient developed bronchopneumonia from which he recovered very slowly. Oedema of the legs became pronounced during the period of recovery with more marked swelling of the right leg than the left suggesting a low grade phlebitis. Metabolism studies had to be discontinued indefinitely. During July and August the urine repeatedly showed many hyalin, granular and cellular casts. The blood

showed moderate secondary anemia, red blood cells, 4.2 millions, Hb. 66 per cent. From August 26th to September 3rd he was given daily doses of 20 gm. of potassium citrate with a reduction in weight from 78.6 to 73.2 kg. and improvement in oedema. Toward the end of October his weight gradually increased again and the oedema of the lower extremities became more marked. Urea clearance test (Van Slyke) October 22, 1929, showed a maximum clearance of about 47 per cent of the normal average. On December 5, 1929 he was readmitted to the metabolism unit for a period of 15 days during which a study of mineral exchanges was carried out as reported in this paper.

At the time of discharge December 21, 1929, the blood count showed red blood cells 5.4 millions, Hb. 82 per cent, white blood cells 10,000. Blood serum protein 4.78 per cent. Urea clearance test (Van Slyke), standard clearance 48 per cent of normal average. Urine, concentrated night specimen 32,000,000 red blood cells for 12 hours (Addis count) very few casts or white blood cells. Protein 4.4 gm. per 12 hours. Specific gravity 1018.

Clinical Impression: Nephritis, subacute hemorrhagic; Hypertension; Heart disease, hypertensive; Bronchopneumonia; ? Phlebitis, right leg.

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EFFECT OF VITAMIN WITHDRAWAL ON THE MONKEY (MACACUS RHESUS)

By

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EXPERIMENTAL and clinical evidence has clearly established that deficiency of vitamin A produces a pathologic entity classified as xerophthalmia. The lowered resistance to infection and the development of lesions appear to be secondary to the development of xerophthalmia. These lesions occur in the cornea, nasal passages, accessory sinuses, middle ear, tongue, and upper respiratory tract.

The experimental production of this condition in eyes and suppurations in the upper respiratory tract of the rat do not prove that similar symptoms will appear in different species of animals under like experimental conditions.

We look to biochemistry to explain many of the mysteries that surround us in the various epidemics of influenza, tonsillitis, mastoiditis or diphtheria. Why does acute sinusitis, tonsillitis, acute laryngitis or mastoiditis prevail in epidemic form and change in type overnight to a greater or lesser degree of virulence and become engrafted on a new area of mucous membrane or lymphoid tissue? If the influence is climatic or microbic, the host with lowered resistance for the particular type of infection succumbs. An understanding of the true factors that determine this resistance may be dependent on a changing biochemistry of the cell with a changing virulence of the micro-organism. From the study of infections in the upper respiratory tract in the rat and monkey this theory has been more clearly substantiated.

This phenomenon, described as xerophthalmia, a disease of the eyes, has been observed by McCollum (1), Drummond (2), and other investigators in the albino rat. Ophthalmia has been observed in dogs (3), rabbits (4), calves (5) and chickens (6). Wolbach and Howe (7) were unable to produce the disease in guinea-pigs.

Evans and Bishop (8) in 1922 observed a change in the epithelial cells of the vaginal wall in rats suffering from lack of vitamin A. Goldblatt and Benischek (9) later showed that this metaplasia likewise was encountered in the tissues of the upper digestive tract and nasal passages. In 1923

Daniels (10) reported that rats afflicted with xerophthalmia often showed suppurations in the nasal cavities and sinuses. This characteristic susceptibility of the rat developing from withdrawal of vitamin A has been confirmed by Macy (11), Sherman (12), Mellanby (13), and Turner (14).

The experimental production of xerophthalmia and infections of the upper respiratory tract in the rodent do not prove that similar symptoms will appear in all species of animals under like experimental conditions. It was thought that similar studies on the monkey might aid in making more clear the relationship of diet to upper respiratory and sinus infection in the human.

Reports on symptoms of the eye disease in monkeys placed on artificial diets free from fat-soluble vitamin A, are rare. McCarrison (15) 1920, placed monkeys on a ration which lacked vitamins A and C. He did not observe an ophthalmia in any of his monkeys but regarded the gastro-intestinal disturbance which developed as due to the absence of these vitamins.

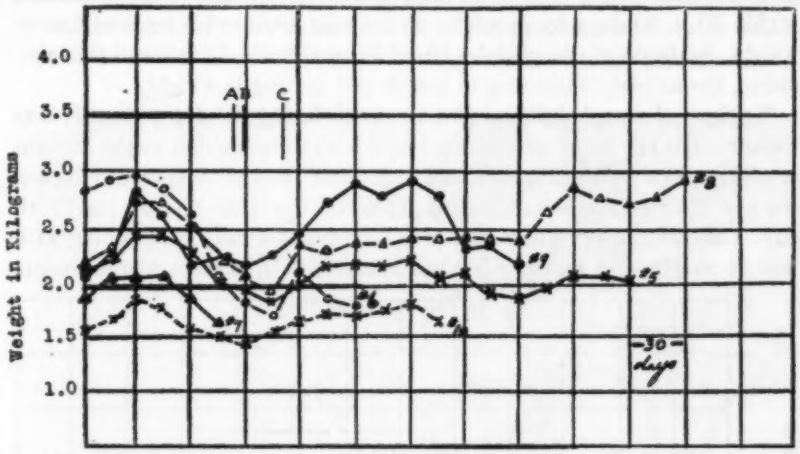
Saiki (16) 1929, studied the liver function of monkeys in which a true fat-soluble deficiency disease was produced. He reports on two animals which showed symptoms of xerophthalmia on the 30th and 47th days of feeding a diet free from vitamin A.

Tilden and Miller (17) 1930, have recently reported observations on 11 monkeys placed on a ration containing 6 to 12 units of vitamin A daily. In no instance did they encounter eye lesions, suppurations in the nasal cavities or in the middle ear. They observed keratinization of epithelial tissue in one or more sites in 9 of the 11 animals. Colitis and gastro-intestinal disorders were the chief lesions observed at autopsy.

The experiments carried out in the laboratories of the Detroit College of Medicine and Surgery include a study on two groups of monkeys. Observations were made on six monkeys (2 to 4 years of age) placed on a diet deficient in vitamins A and C (McCarrison's) followed by a diet deficient in vitamin A (Saiki's) until death. Vaginal smears were taken three times a week and the results recorded with the menstrual dates. The second group consisted of eight monkeys (1 to 2 years of age). These monkeys received the Saiki diet only. In this group a histological study was made of tissues from the bladder, spleen, intestines, trachea, thyroid, salivary glands, lungs and eyelids. The sections were taken immediately after the animals succumbed. Observations were also made on eight control monkeys (4 with each group) which received diets with adequate amounts of vitamins. Vitamin E was not supplied in any of the diets.

The results of these experiments show that monkeys on a diet deficient

CHART NO. 1. GROWTH CURVES OF MONKEYS PLACED ON VITAMIN-A-DEFICIENT DIET TEST.

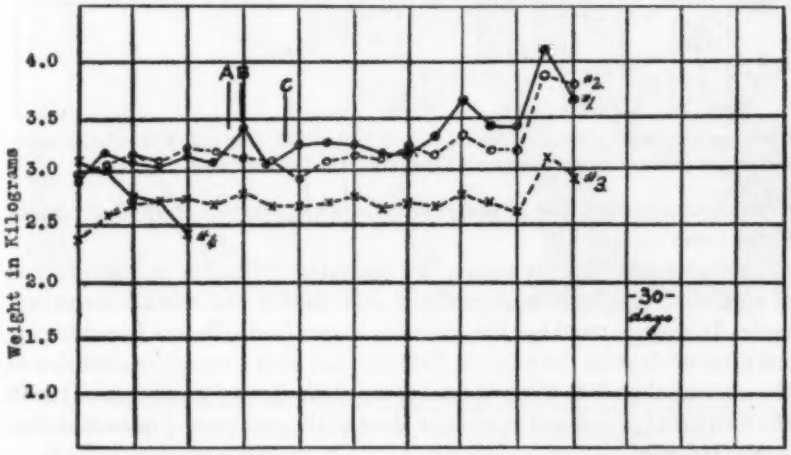


A.—Animals showed weakness and paresis of rear limbs. Purified orange juice added to diet to provide vitamin C.

B.—Mature females showed continued presence of cornified cells in vaginal smears. Menstruation ceased or interval was greatly prolonged.

C.—Changed from McCarrison's diet to diet formulated by Saiki.

CHART NO. 2. GROWTH CURVES OF MONKEYS PLACED ON COMPLETE RATION CONTROLS.



A.—No loss in weight, weakness or paresis of rear limbs. Oranges were included in complete ration from beginning of experiment.

B.—Mature females did not show continued presence of cornified cells in vaginal smears. Menstruation continued regularly.

C.—Changed from McCarrison's diet to diet formulated by Saiki.

in both vitamins A and C, develop a scurvy-like condition, indicated within 70 to 80 days by paralysis of the rear limbs, with marked loss in weight. Addition of orange juice (freed from vitamin A) relieved this condition, the animals improving in health and gaining in weight.

No signs of xerophthalmia were observed during the depression. It was thought that the bread or milk used in the McCarrison diet might contain small traces of vitamin A, sufficient to prevent the animal from developing the eye disease. The animals were placed on the Saiki diet on the 110th day. They lived on this diet from 1 to 10 months, finally succumbing with loss of weight and gastro-intestinal symptoms. Abscesses of the tongue

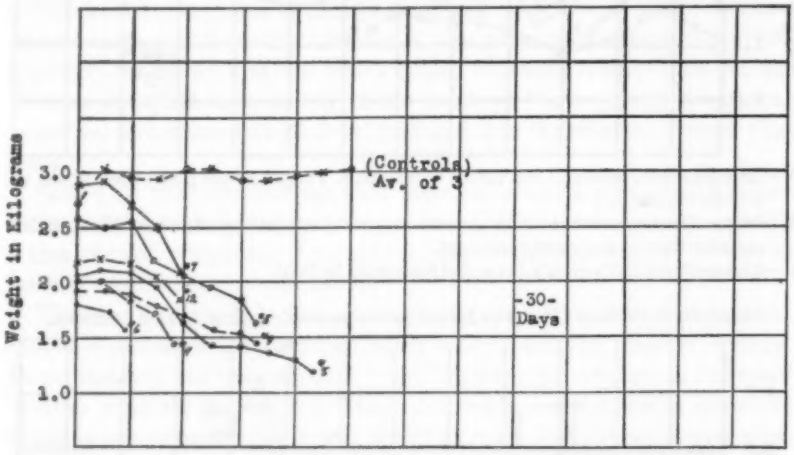


CHART 3. Control animals received 3% C. L. O. in their diet. Curves 5 to 12 inclusive represent growth curves of young monkeys on Saiki Vitamin A deficient diet.

were encountered in one of these animals, while another showed abscesses of the lower molars.

Vaginal smear records showed no consistent variation in the presence of epithelial, leukocyte and cornified cells during the normal menstrual cycle. In mature monkeys the presence of cornified cells was found to persist after 60 days on the vitamin-deficient diet with a complete cessation of the menstrual period. Control mature monkeys showed no abnormalities in the menstrual period and at no time showed the continued presence of desquamated cells.

The younger monkeys lived from 3 to 5 months on the vitamin A-deficient diet, succumbing with symptoms identical to those found in the older monkeys. In general, death of these monkeys on vitamin A-deficient ra-

tions was preceded by loss in weight accompanied with a severe diarrhea terminating 'n a mucous-like exudate. Postmortem examination showed evidence of intestinal inflammation with marked enteritis and dilatation of

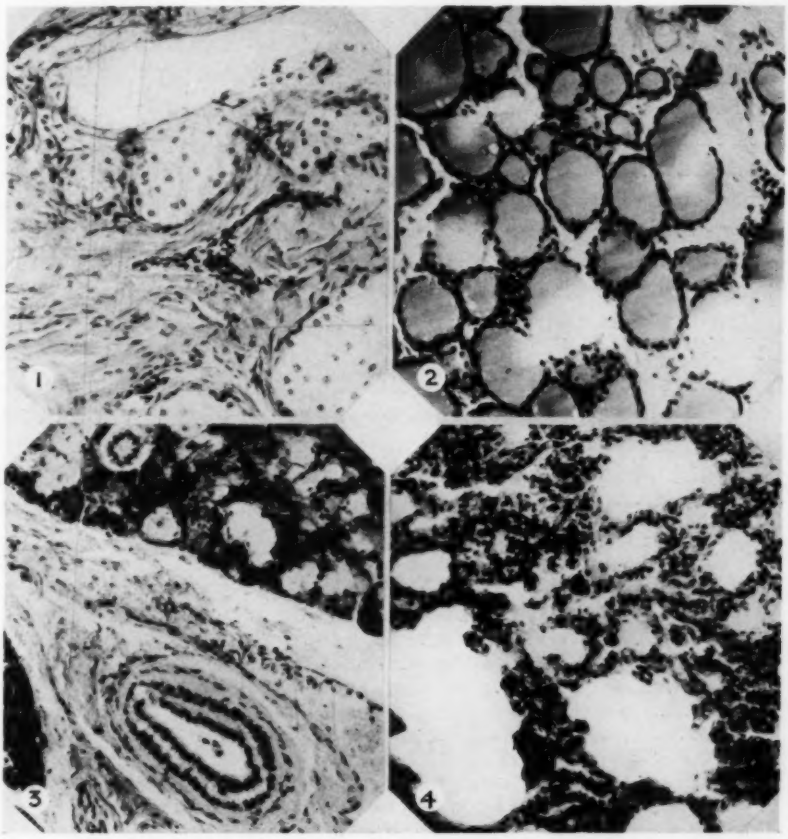


FIGURE 1.

No. 1. Eyelid of monkey No. 8. × 100. Showing a follicular process with local aggregation of lymphocytic cells beneath the mucous membrane. No evidence of metaplasia is evident.

No. 2. Thyroid gland of monkey No. 8. × 100. Showing no evidence of metaplasia or keratinization of the epithelial cells.

No. 3. Salivary gland of monkey No. 8. × 100. Showing no evidence of metaplasia or hyalinization of the epithelial cells.

No. 4. Lung of monkey No. 8. × 100. Showing no evidence of bronchial pneumonia or metaplasia of epithelial cells. Atelectasis is evident in a few small areas.

the stomach. Xerophthalmia was not observed. The upper respiratory tract, nasal cavities and middle ear were free from suppuration in all of the deficient animals.

Histological examination of the 8 younger monkeys and monkey No. 8 of the older group, showed no signs of keratinization or hyalinization of the epithelia in the tissue studied.

Most of the control monkeys maintained their weight, with minor fluctuations, throughout the experimental period. Three of the control monkeys died, one showing bronchial pneumonia, the other two symptoms of tuberculosis with abscessed spleen, liver and lungs. The latter picture was not observed in any of the animals succumbing from lack of vitamin A.

In conclusion, it appears that withdrawal of vitamin A from the monkey does not develop a characteristic susceptibility toward infections of the upper respiratory tract. Xerophthalmia, at least, is difficult to produce and no definite change in the tissues of the upper digestive tract are observed.

Thus it seems that infections of the upper respiratory tract brought about by experimental diets are encountered most frequently when that portion of the tract is affected by a change in cell structure.

We wish to express our thanks to Dr. A. L. Amolsch who was kind enough to examine the microscopic tissue preparations and to Dr. Burt R. Shurly whose interest has made this work possible.

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THE HEMOGLOBIN CONTENT OF THE BLOOD OF DAIRY CATTLE*

By

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ONE of the problems encountered in studying the efficiency of milk alone as a feed for dairy calves was nutritional anemia. In interpreting results, it was important to know the hemoglobin content of the blood of normal dairy calves. No work could be found reported in the literature on this subject. Dimock and Thompson (1) report an average of 8.96 grams of hemoglobin in 100 cc. of blood in an examination of 20 head of cattle of the four major dairy breeds in the Cornell dairy herd. The maximum was 12.75 grams and the minimum 6.75 grams. However, no determinations were made on calves. In order to secure such information, the hemoglobin content of the blood of the calves in four dairy herds was determined and at the same time a study of all ages was included. Determinations were made in the college herd consisting of the four major breeds of dairy cattle, in the Holstein herd at the Topeka State Hospital, the Holstein herd at the Osawatomie State Hospital, and in a privately owned herd of Guernsey cattle.

In all a total of 335 determinations were made on 297 head.

METHOD

The acid hematin method (2) was used for the determination of the hemoglobin. A 0.05 cc. sample of blood was taken from the ear vein by means of a capillary pipette and was introduced into 10 cc. of tenth normal hydrochloric acid. After the hemoglobin color had fully developed, the sample was matched against a standard. This standard was made up from blood that had been standardized by means of the Van Slyke method (3) for determining the combined oxygen of the blood. Although perhaps not necessary, a new standard was prepared each week.

The results from the four herds are tabulated in Table I according to the four groups, cows, heifers, calves, bulls. Included in the cow group

* Contribution No. 76 from the Department of Dairy Husbandry and contribution No. 159 from the Department of Chemistry.

† Acknowledgment is made of the assistance given by Dr. J. L. Hall of the Department of Chemistry in the chemical analyses in this project.

TABLE I
HEMOGLOBIN CONTENT OF THE BLOOD OF DAIRY CATTLE

Group	No. of animals	Grams of hemoglobin per 100 c.c. blood		Standard deviation
Cows	103	10.94	±.1034	1.538
Heifers	59	11.71	±.1089	1.241
Calves	111	10.46	±.1059	1.654
Bulls	24	11.92	±.2612	1.893
Average		10.96	±.0638	1.635
Maximum	3 Yr. Ayr. heifer	16.50		
Minimum	1 Mo. Guer. bull	5.90		

are all females that had calved at least once, in the heifer group, females from 5 to 28 months of age that had not yet calved; in the calf group, animals of both sexes up to 16 months of age that were still maintained with the calf herd, while in the bull classification were included all male animals over 9 months of age. The calf group necessarily included some animals from both the bull and the heifer groups.

As noted in Table I, the hemoglobin content of the blood is rather constant in dairy animals. The individuality of the animal seemed to be the most important factor in causing variations from the normal.

The mean for all determinations was 10.98 ± .064 grams of hemoglobin in the 100 cc. of blood. The group of 24 bulls varying from nine months to fourteen years of age gave the highest reading, followed in close order by the heifer, cow and calf groups. The lowest value of 5.90 grams of hemoglobin in 100 cc. of blood obtained in a single individual was upon a one month old Guernsey bull owned in the private herd. This fact, together with the facts of the low average hemoglobin content of the blood of this Guernsey herd as well as that of the Guernseys in the College herd and the lower average for all Guernseys shown in Table II, suggests a

TABLE II
SUMMARY OF DATA BY BREEDS

Breed	No. of Animals	Grams Hb in 100 cc. blood	
Jersey	13	11.60	±.2039
Guernsey	77	10.34	±.1255
Ayrshire	32	11.44	±.1786
Holstein	175	11.10	±.0812

breed difference. However, it is noted that the Holstein herd at the Osawatomie State Hospital showed the lowest hemoglobin content of any herd. It may also be noted in Table III that there is a greater difference between the same group in the respective herds than between groups within a herd. This is rather difficult to explain and suggests further study as to just why such differences between herds should exist.

TABLE III

SUMMARY OF ALL DATA BY HERDS

Group	College Herd Hol.-Ayrs.-Guer.-Jers.		Topeka State Hospital Hol.		Osawatomie State Hospital Hol.		Guernsey Herd	
	No. An.	Hb	No. An.	Hb	No. An.	Hb	No. An.	Hb
Cows	38	11.62	26	11.94	21	10.43	28	9.85
Heifers	19	11.99	30	12.10	10	10.46	—	—
Calves	30	10.38	18	12.68	39	9.46	24	10.13
Bulls	8	12.93	4	12.99	9	10.61	4	10.80
Summary	95	11.36	78	12.27	79	9.97	56	10.05

The maximum value of 16.50 grams of hemoglobin in the 100 cc. of blood obtained in a single individual was found in the case of a three year old Ayrshire heifer in milk in the College herd. In regard to the groups, the Holstein calf herd at the Osawatomie State Hospital consisting of 39 animals gave the minimum value of 9.46 grams of hemoglobin. A group of four Holstein bulls at the Topeka State Hospital averaged 12.99 grams of hemoglobin which was the maximum for the groups. These differences are hardly sufficiently great to be significant.

There seemed to be no significant relationship between the age of the animal and the hemoglobin content of the blood. This fact is brought out in Table IV which shows the summary of the determinations on all cattle grouped in 5-month intervals.

In order to note any variation in the hemoglobin content of the blood from day to day, determinations were made daily on a group of three animals for an eleven day period. These animals showed very little variation throughout the period. The mean for the group for the period was 10.91 grams of hemoglobin, with a standard deviation of 0.416.

Similar results were obtained on a group of five heifers of the four major breeds that were fasted for fourteen days in a blood sugar deter-

TABLE IV

VARIATION OF HEMOGLOBIN WITH AGE

Age mos.	No. of animals	Hb	Age mos.	No. of animals	Hb
3	54	10.53	73	2	12.23
8	58	10.52	78	6	10.91
13	32	11.21	83	4	11.62
18	14	11.76	88	6	10.08
23	23	11.85	93	8	11.27
28	14	11.73	98	3	10.18
33	12	11.49	103	2	9.59
38	9	11.21	108	2	10.72
43	7	9.98	113	2	11.04
48	6	11.26	118	4	11.36
53	5	10.77	123	2	10.76
58	11	11.18	128	2	11.36
63	4	10.73	133	2	11.95
68	3	11.64			

mination experiment. The mean for this group for the period was 12.98 grams of hemoglobin per 100 cc. of blood, with a standard deviation of 0.721.

CONCLUSIONS

1. The mean hemoglobin content of the blood of dairy cattle in 335 determinations on 297 head was $10.96 \pm .064$ grams of hemoglobin in the 100 cc. of blood.

2. Such factors as breed, age, and prolonged fasting did not seem to affect appreciably the hemoglobin content of the blood and no significant individual variation was observed from day to day.

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FACTORS WHICH DETERMINE THE CONCENTRATION OF CALCIUM AND OF INORGANIC PHOSPHORUS IN THE BLOOD SERUM OF RATS

Second Paper

By

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INTRODUCTORY REMARKS

IT IS customary to speak of a normal concentration of calcium or of inorganic phosphorus in serum, and if normal individuals subsisting on a well balanced diet are chosen the concentration of these components in serum will be found to be singularly constant (1). Thus, the serum calcium concentration of normal infants free from clinical or x-ray evidence of rickets or of clinical evidence of tetany is found to be 10 ± 1 mg. per 100 cc. of serum, while the inorganic phosphorus is 5 ± 0.5 mg. Measurable deviations from the normal have been found in disease and may be sustained over a long period of time but where such deviations are marked, clinical symptoms soon appear and in some instances are characteristic of the particular inorganic deficiency. A marked decrease in serum calcium has been observed in infantile (2) and parathyroid (3) tetany and in chronic nephritis, particularly the type designated as nephrosclerosis (4). In cases of chronic parenchymatous nephritis with edema and low serum protein there is a parallelism between the reduction in serum protein and in serum calcium (5). A moderate decrease of serum calcium may follow operations on the thyroid gland and has been ascribed to incidental damage to the parathyroids (6). Reductions in serum calcium concentration have been reported in rickets without tetany (7), in osteomalacia (8), in coeliac disease (9), in sprue (10), and recently, in pneumonia (11). Transient increases in serum calcium may be produced in normal individuals or animals by the oral administration of large amounts of soluble calcium salts or the intravenous injection of smaller amounts (12). The repeated injection of parathyroid extract produces a marked hypercalcaemia with vomiting, muscular atony, profound depression, anorexia,

* Deceased.

ataxia, bloody stools, and other symptoms (13). Hypercalcaemia may follow the administration of toxic doses of irradiated ergosterol (14).

The concentration of inorganic phosphorus in blood serum is not so constant as that of calcium. The low inorganic phosphorus of severe infantile rickets and of the experimental form of this disease, is well known. Gerstenberger and his collaborators have reported low values in pneumonia (15), in the absence of demonstrable rachitic changes in the bones or in spite of anti-rachitic therapy. A marked elevation of the inorganic phosphorus level in serum is a common finding in nephrosclerosis, where it is often accompanied by a proportional decrease of the serum calcium, a reciprocal relationship which has been frequently observed (16).

In 1922 (17), we reported some observations on a series of rats which had been fed on diets that allowed normal growth for at least four generations and a maximum longevity. The value obtained for serum calcium in these animals was 10 ± 0.5 mg. in 100 cc. of serum; for inorganic phosphorus 7.7 ± 0.7 mg. Two basal diets were used, one deficient in calcium and the other in phosphorus. To these basal diets various additions, including cod liver oil and other fish oils, vegetable oils and butter fat, were made. In some experiments, the calcium phosphorus discrepancy was equalized, by the addition of salts of calcium or phosphorus or an organic phosphorus compound. Other groups of animals were exposed to radiations emanating from various sources and containing varying amounts of ultra violet light of wave lengths less than 3000 Ångstrom units.

Our conclusions may be summarized as follows:

The concentration of calcium and of inorganic phosphorus in the serum is determined by at least four factors, namely, the calcium content of the diet, its total phosphorus, the ratio of these two components in the diet and the amount of vitamin D preformed or produced by irradiation. Without the balancing effect of vitamin D in the form of cod liver oil, butter fat, or ultra violet light the calcium and phosphorus concentration of the serum varies directly with the concentration of the same element in the diet. A discrepancy between the concentration of these two elements in the diet is reflected as a similar discrepancy in the blood serum.

In 1923 a contribution by Park and his collaborators (18), appeared bearing on the same point. Rats were fed a basal ration low in calcium, phosphorus and fat soluble organic factor (calcifying factor). A number of diets were prepared by adding different amounts of calcium carbonate or disodium phosphate or both to this basal diet. The experimental diets were fed for 33 to 35 days. At the end of this period the control animals were killed and calcium and inorganic phosphorus determined on the serum. Two per cent cod liver oil was added to the diet of the remaining animals and these were also sacrificed at the end of ten days. The cod

liver oil addition increased the calcium concentration in each instance. With the low calcium, low phosphorus diet the serum calcium was increased but the inorganic phosphorus remained practically unaltered. The marked discrepancy between calcium and phosphorus in the diet was reflected in the concentration of these elements in the blood of the control animals. A low calcium/phosphorus ratio in the diet yielded a similar ratio in the serum and vice versa. This discrepancy was partly equalized after cod liver oil feeding. To quote Dr. E. A. Park:

The experiments demonstrate in a striking manner the regulatory power of cod liver oil on the calcium and phosphorus metabolism of the organism. The changes in the concentration of the calcium and inorganic phosphorus of the blood serum which occurred under the influence of cod liver oil were striking in degree. Further, cod liver oil not only acts as a regulator of the calcium and phosphorus metabolism, but also permits the organism to operate with greatly increased economy. In the presence of calcium or phosphorus starvation, cod liver oil enables the animal to get along as if the calcium or the phosphorus were supplied in sufficient or almost sufficient quantity in the diet. It is necessary to suppose that cod liver oil brings about maximal utilization of the minimal quantities of calcium or phosphorus in the diet. It greatly reduces waste of these elements and, therefore, must bring about maximal absorption from the alimentary tract. Obviously, cod liver oil makes the metabolic processes of the body, in respect to calcium and phosphorus, vastly more efficient.

Drs. Park, Guy and Powers recognized the regulatory power of cod liver oil on calcium and phosphorus metabolism, a power which was exerted by virtue of the more efficient utilization of these elements under the influence of the oil.

In 1924 and 1925, we had the opportunity of analyzing the sera of a large number of rats that were the subject of an experiment by Prof. McCollum and Miss Simmonds, to determine the effect upon bone development, growth, etc., of quantitative variations in the concentration of calcium, inorganic phosphorus and calcifying factor in the diets of rats. Through the courtesy of Prof. McCollum and Miss Simmonds we are reporting these results. Although our knowledge of the process of calcification, of rickets and other bone diseases and of the factors which determine inorganic equilibria in serum has been substantially enhanced since this work was completed, it is believed that the results are of sufficient interest to justify their publications at this late date.

Experimental

"The rats were fed a basal diet consisting of the following:[1]

Rolled oats	40.0 gm.
Gelatin	10.0 "

[1] The experiments were carried out in Prof. McCollum's laboratory in the Department of Chemical Hygiene, of the School of Hygiene, of The Johns Hopkins University. At the end of each experiment the animals were weighed and then sent to us for the analysis of their blood serum.

Wheat gluten	7.0 "
NaCl	1.0 "
KCl	1.0 "
Dextrin	41.0 "

This diet contained 0.181 grams of phosphorus and 0.045 of calcium in 100 grams of food. The proportion of calcium to phosphorus was varied by adding $CaCO_3$ or KH_2PO_4 or both. The diets were fed for approximately six weeks. Vitamin D was fed at different levels by using 1 per cent butter fat, 10 per cent butter fat and 2 per cent cod liver oil. The calcium of the diet was varied from 0.14 to 1.24, the phosphorus from 0.2 to 1.2, grams per 100 grams of food."

At the end of the experimental period the animals were sacrificed, and the sera pooled and analyzed for calcium and inorganic phosphorus. Hemolyzed sera were discarded. Determinations were all done in duplicate. The Kramer-Tisdall method was used for the calcium determinations[2] and the Briggs-Bell-Doisey method for inorganic phosphorus. The animals were weighed at the beginning and at the end of each experiment. The weight gain was recorded for each animal and the average gain for the group was calculated.

Series 1

In this series of experiments, the butter fat in the diet was maintained at a 1 per cent level. The calcium content was varied from 0.14 to 1.24 per cent and the total phosphorus from 0.2–1.2 per cent. With the lowest calcium concentration in the diet that of the serum remains at the low level of 5.7 ± 1 mg. Lower values seem to be incompatible with life. These animals either lost weight or gained very little.

As the calcium of the diet increased so did that of the serum until at a calcium level of 1.04 per cent in the diet, that of the serum was normal. When phosphorus was fed at a level of 0.8 per cent it was necessary to increase the calcium of the diet to 1.24 per cent to attain a normal concentration of serum calcium. As the calcium concentration in the diet and that in the serum increased, the inorganic phosphorus of the serum decreased. With fixed calcium intake the inorganic phosphorus of the serum rose parallel with that in the diet. Thus, with a calcium of 1.24 and phosphorus of 0.2 per cent the serum phosphorus was 3.0 mg. per 100 cc. of serum. With the same calcium concentration in the diet and 0.8 per cent phosphorus the serum inorganic phosphorus was 8.7 mg. When the phosphorus of the diet was raised to 1.2 that of the serum was 9.7 mg. per cent.

In the presence of inadequate amounts of vitamin D there seemed to be

[2] The samples were allowed to stand over night for complete precipitation of the calcium oxalate.

TABLE I

EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.2%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca*	Serum P*	Weight gain gm.
P=0.2 Ca=0.14	0.72	5.4	6.5	21
P=0.2 Ca=0.24	1.22	6.2	5.7	31
P=0.2 Ca=0.64	3.22	9.7	5.6	35
P=0.2 Ca=1.04	5.23	10.2	3.9	25
P=0.2 Ca=1.24	6.22	10.2	3.0	20

* Concentrations expressed in mg. per 100 cc. of serum.

TABLE II

EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.4%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.4 Ca=0.14	0.36	5.3	11.2	26
P=0.4 Ca=0.24	0.61	5.5	8.8	21
P=0.4 Ca=0.44	1.11	6.5	9.5	37
P=0.4 Ca=0.64	1.61	6.9	8.7	28
P=0.4 Ca=0.84	2.11	10.0	6.0	42
P=0.4 Ca=1.04	2.61	11.0		40
P=0.4 Ca=1.24	3.11	11.0	3.5	25

TABLE III

EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.6%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.6 Ca=0.14	0.24	4.6	11.3*	14
P=0.6 Ca=0.24	0.40	5.8	10.1*	18
P=0.6 Ca=0.44	0.74	5.6	12.2*	20
P=0.6 Ca=0.64	1.07	7.3	11.6	33
P=0.6 Ca=0.84	1.4	8.8	8.8	33
P=0.6 Ca=1.04	1.75	10.0	7.4	40
P=0.6 Ca=1.24	2.07	10.9	6.5	28

* Tetany

TABLE IV

EFFECT OF BUTTER FAT 1%, PHOSPHORUS 0.8%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.8 Ca=0.24	0.3	6.4	10.2	13
P=0.8 Ca=0.44	0.5	7.2	10.5	6.0
P=0.8 Ca=0.64	0.81	6.8	11.3	29
P=0.8 Ca=0.84	1.05	8.4	8.0	27
P=0.8 Ca=1.04	1.30	8.3	8.7	30
P=0.8 Ca=1.24	1.55	10.5	8.7	23

TABLE V

EFFECT OF BUTTER FAT 1%, PHOSPHORUS 1.0%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.0 Ca=0.14	0.14	6.7	11.3	8.0
P=1.0 Ca=0.24	0.24	6.9	11.3	6.0
P=1.0 Ca=0.44	0.44	6.3	10.4	7.0
P=1.0 Ca=0.64	0.64	7.0	10.4	10.0
P=1.0 Ca=0.84	0.84	7.2	9.1	18.0
P=1.0 Ca=1.04	1.04	8.0	8.0	20.0
P=1.0 Ca=1.24	1.24	8.4	7.8	30.0

TABLE VI

EFFECT OF BUTTER FAT 1%, PHOSPHORUS 1.2%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.2 Ca=0.14	0.12	5.6	11.5	−5.0
P=1.2 Ca=0.24	0.20	5.9	11.4	+5.0
P=1.2 Ca=0.44	0.37	6.4	9.9	11.0
P=1.2 Ca=0.64	0.54	7.4	9.9	18.0
P=1.2 Ca=0.84	0.7	7.2	9.7	21.0
P=1.2 Ca=1.24	1.04	8.1	—	33.0

an antagonism between calcium and inorganic phosphorus. A high concentration of one element depressed the concentration of the other component in the serum. With the calcium of the diet constant the inorganic phosphorus of the serum fluctuated in a manner parallel with that in the diet. The serum calcium behaved in a similar manner with respect to the

TABLE VII

EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.2%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.2 Ca=0.145	0.72	7.0	—	31
P=0.2 Ca=0.24	1.22	9.3	8.3	52
P=0.2 Ca=0.44	2.22	10.4	7.0	37
P=0.2 Ca=0.64	3.22	10.9	5.9	41
P=0.2 Ca=0.84	4.22	10.5	3.9	16
P=0.2 Ca=1.04	5.23	10.5	3.5	12
P=0.2 Ca=1.24	6.22	11.0	3.9	12

food calcium. The amount of *available* calcium seems to decrease as the diet phosphorus increases; the same is true of phosphate with increasing amounts of diet calcium. With a diet containing from 0.14 to 0.44 per cent calcium and 0.6 per cent phosphorus a serum calcium of 4.6 to 5.8 mg. was produced with a serum inorganic phosphorus of 10.1 to 12.2 mg. per cent and the animals developed tetany.

SERIES 2

A comparison of Tables VII to XII inclusive with the previous tables reveals the effect of the increased content of butter fat in the diet. The effect is presumably due to the increased content of vitamin D in this

diet although the experiments supply no proof for this assumption. With less than optimal amounts of calcium in the diet the calcium level in the serum is higher than in the previous experiments, but still subnormal. Thus, a diet containing 0.24 per cent calcium and 0.2 per cent phosphorus with 10 per cent butter fat gives a serum calcium only slightly less than

TABLE VIII

EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.4%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.4 Ca=0.14	0.36	6.2	11.6	29
P=0.4 Ca=0.24	0.61	7.6	12.0	46
P=0.4 Ca=0.44	1.11	11.0	9.2	42
P=0.4 Ca=0.64	1.61	10.6	11.1	52
P=0.4 Ca=0.84	2.11	10.6	9.5	52
P=0.4 Ca=1.04	2.61	11.0	7.9	46
P=0.4 Ca=1.24	3.11	11.4	9.4	35

normal (9.3 mg. per cent) whereas with 1 per cent butter fat the serum calcium concentration is only 6.2 mg. Similarly the serum inorganic phosphorus level is greater when more butter fat is incorporated in the diet.

SERIES 3

In this series of experiments cod liver oil was fed with the diet at a 2 per cent level in place of butter fat. With the exception of the last four analyses in Table XIII, where a measurable hypercalcemia seems to have been produced by feeding a diet rich in calcium salts and low in phosphorus, the calcium concentration of the serum maintained a singularly constant level in spite of an approximately nine-fold increase in the

TABLE IX
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.6%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.6 Ca=0.14	0.24	6.0	9.9	40
P=0.6 Ca=0.24	0.40	8.2	9.3	42
P=0.6 Ca=0.44	0.74	11.1	10.6	54
P=0.6 Ca=0.64	1.07	11.1	9.8	59
P=0.6 Ca=0.84	1.4	10.2	9.2	59
P=0.6 Ca=1.04	1.75	10.5	8.8	60
P=0.6 Ca=1.24	2.07	10.2	8.5	46

TABLE X
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 0.8%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.8 Ca=0.14	0.18	5.9	10.4	23
P=0.8 Ca=0.24	0.30	8.5	9.6	30
P=0.8 Ca=0.44	0.55	11.0	9.4	41
P=0.8 Ca=0.64	0.80	10.7	8.8	47
P=0.8 Ca=0.84	1.05	11.4	10.5	48
P=0.8 Ca=1.04	1.30	11.0	9.3	50
P=0.8 Ca=1.24	1.55	10.5	8.8	55

TABLE XI
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 1.0%. VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.0 Ca=0.14	0.14	5.6	13.7	27
P=1.0 Ca=0.24	0.24	8.0	11.0	30
P=1.0 Ca=0.44	0.44	10.7	9.9	43
P=1.0 Ca=0.64	0.64	10.9	9.7	45
P=1.0 Ca=0.84	0.84	11.5	9.0	58
P=1.0 Ca=1.04	1.04	10.7	8.8	55

TABLE XII
EFFECT OF BUTTER FAT 10%, PHOSPHORUS 1.2%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.2 Ca=0.14	0.12	5.5	—	8.0
P=1.2 Ca=0.24	0.20	7.0	9.8	28
P=1.2 Ca=0.44	0.37	8.9	9.9	48
P=1.2 Ca=0.64	0.54	9.9	9.9	49
P=1.2 Ca=0.84	0.70	10.7	9.9	54
P=1.2 Ca=1.04	0.87	10.9	10.0	55
P=1.2 Ca=1.24	1.04	11.8	10.6	53

TABLE XIII
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.2%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.2 Ca=0.14	.72	10.0	11.8	63
P=0.2 Ca=0.24	1.20	10.6	13.1	53
P=0.2 Ca=0.44	2.22	10.9	9.0	52
P=0.2 Ca=0.64	3.22	12.6	6.8	42
P=0.2 Ca=0.84	4.22	13.2	7.5	23
P=0.2 Ca=1.04	5.22	13.3	8.1	13
P=0.2 Ca=1.24	6.22	13.6	7.5	10

TABLE XIV
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.4%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.4 Ca=0.14	0.36	10.7	9.3	72
P=0.4 Ca=0.24	.61	10.6	9.0	75
P=0.4 Ca=0.44	1.11	10.6	8.6	81
P=0.4 Ca=0.64	1.61	10.6	9.3	62
P=0.4 Ca=1.24	3.11	12.0	9.1	77

calcium of the diet and a six-fold variation of the phosphorus in the diet. Similarly disregarding the hyperphosphataemia in the first three analyses in Table XV and four in Table XVI we see here also a remarkable constancy of the inorganic phosphorus in the serum.

Growth

The rats were weighed at the beginning and end of the experimental period. The recorded gain in weight represents an average of the weight gain of five to eight animals. In most of the experiments eight animals were weighed and the increase averaged. Marked deviations from the average were observed in single litters.

With a diet containing only 1 per cent butter fat and 0.14 per cent calcium the gain in weight decreased as the phosphorus in the diet was increased. With moderate amounts of calcium in the diet, *i.e.*, 0.64 per cent, the weight gain again dropped as the phosphorus of the diet increased, the increase of food phosphorus being equal to a drop in diet calcium. With the addition of 10 per cent butter fat to the diet there was a distinct improvement in the weight gain for each group. The substitution of cod liver oil still further improved the weight gain of all the animals.

In the experiment summarized in Table XIII we find a progressive decline in weight gain as the calcium increased in the diet. A similar phenomenon though less striking is seen in Table VII in the corresponding experiment with butter fat 10 per cent. In these experiments the low food phosphorus in the presence of a high calcium intake acts as the limiting factor for growth and the higher the calcium intake with a fixed concentration of phosphorus in the diet the poorer the growth.

Failure to gain weight due to low calcium intake is more readily overcome by addition of cod liver oil than is that due to inadequate phosphorus intake. Thus, with a diet calcium of 0.14 per cent and a diet phosphorus of 0.2 the weight gain with 1 per cent butter fat is 21 gm., that with 10 per cent butter fat is 31 gm. while that with 2 per cent cod liver oil is 63 gm. Whereas with a calcium intake of 1.24 per cent and a phosphorus in the diet 0.2 per cent there is very little difference in weight gain whether 1 per cent butter fat, 10 per cent butter fat or 2 per cent cod liver oil is used, namely, 20, 12, 10 gms. respectively. That lack of *available* phosphorus in the diet is the limiting factor in growth even in the presence of 10 per cent butter fat or 2 per cent cod liver oil is indicated by the fact that a marked improvement in weight follows the addition of phosphorus to the diet, other factors remaining constant. An increase of but 0.2 per cent phosphorus in the diet has a striking effect.

TABLE XV
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.6%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.6 Ca=0.14	.24	11.2	12.2	54
P=0.6 Ca=0.24	.40	11.2	11.1	73
P=0.6 Ca=0.44	.74	10.3	10.8	55
P=0.6 Ca=0.64	1.07	10.2	9.0	87
P=0.6 Ca=0.84	1.40	10.5	8.5	55
P=0.6 Ca=1.04	1.74	10.8	9.1	73
P=0.6 Ca=1.24	2.07	10.6	8.3	65

TABLE XVI
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 0.8%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=0.8 Ca=0.14	.18	9.6	9.5	60
P=0.8 Ca=0.24	.30	10.2	10.	65
P=0.8 Ca=0.44	.55	10.3	11.3	55
P=0.8 Ca=0.64	.80	10.2	10.7	52
P=0.8 Ca=0.84	1.05	10.2	11.4	56
P=0.8 Ca=1.04	1.30	10.9	8.4	70
P=0.8 Ca=1.24	1.55	10.2	8.5	83

TABLE XVII
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 1.0%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.0 Ca=0.14	0.14	10.2	7.5	71
P=1.0 Ca=0.24	0.24	10.0	8.0	65
P=1.0 Ca=0.44	0.44	9.8	7.6	70
P=1.0 Ca=0.64	0.64	10.0	7.5	73
P=1.0 Ca=0.84	0.84	10.2	8.5	83
P=1.0 Ca=1.04	1.04	10.3	8.3	68

TABLE XVIII
EFFECT OF COD LIVER OIL 2%, PHOSPHORUS 1.2%, VARIABLE CALCIUM OF DIET ON CA AND P OF SERUM

Diet	Ca/P in diet	Serum Ca	Serum P	Weight gain gm.
P=1.2 Ca=0.14	.12	10.7	9.5	53
P=1.2 Ca=0.24	.20	10.3	8.8	53
P=1.2 Ca=0.44	.37	10.9	9.3	69
P=1.2 Ca=0.64	.54	10.7	8.4	61
P=1.2 Ca=0.84	.70	10.5	8.2	71
P=1.2 Ca=1.04	.87	10.2	8.5	67
P=1.2 Ca=1.24	1.04	10.8	8.9	58

TABLE XIX

EFFECT OF VARIABLE AMOUNTS OF CALCIFYING FACTOR WITH CALCIUM AND PHOSPHORUS IN THE DIET AT CONSTANT AND MINIMAL LEVELS

Diet	Vitamin	Ca	P
P=0.181 Ca=0.045	C. S. oil 10	6.2	9.3
P=0.181 Ca=0.045	B. Fat 1.0	5.8	10.0
P=0.181 Ca=0.045	No Fat	6.0	9.1
P=0.181 Ca=0.045	B. Fat 10.0	6.0	12.0
P=0.181 Ca=0.045	C. L. O. 2%	7.2	5.6

C. S.=Cotton Seed Oil B. F.=Butter Fat C. L. O.=Cod Liver Oil

Series 4

In this series of experiments diets containing minimal amounts of calcium and very small amounts of phosphorus were fed. These diets were fed either alone or with a supplement containing a variable amount of vitamin D. In this experiment neither cotton seed oil nor butter fat fed at 1 or 10 per cent level had any influence upon the serum calcium. It is significant that in the presence of minimal amounts of calcium and phosphorus in the diet enough phosphorus was absorbed to produce hyperphosphataemia in the absence of calcifying factor but the serum calcium remained low. Two per cent cod liver oil, however, not only raised the serum calcium but in doing so depressed the inorganic serum phosphorus.

Series 5

In this series of experiments, the calcium and phosphorus concentrations in the diet were maintained at a constant level as was the calcium/phosphorus ratio but the fat soluble factor was varied. When this Ca/P ratio in the diet is 1.5, the same calcium and inorganic phosphorus prevails in the serum irrespective of the amount of organic factor in the diet. Although the calcium/phosphorus ratios in the diets in this series of experiments varied from Ca/P=0.4 to Ca/P=6.0, 2 per cent cod liver oil added to the diet served to maintain a normal concentration of calcium and inorganic phosphorus in the blood serum.

TABLE XX
EFFECT OF VARIABLE VITAMIN D IN DIET

Vitamin D	Serum Ca	Serum P	Weight gain gm.
Diet Ca/P=0.4	P=1.0	Ca=0.44	
B.F. 1	6.3	10.4	7.0
B.F. 10	10.7	9.9	43
C.L.O. 2	9.8	7.6	70
Diet Ca/P=1.0	P=1.0	Ca=1.04	
B.F. 1	8.0	8.0	20
B.F. 10	10.7	8.8	55
C.L.O. 2	10.3	8.3	68
Diet Ca/P=1.5	P=0.8	Ca=1.24	
B.F. 1	10.5	8.7	23
B.F. 10	10.5	8.8	55
C.L.O. 2	10.2	8.5	82
Diet Ca/P=6.0	P=0.2	Ca=1.24	
B.F. 1	10.2	3.0	20
B.F. 10	11.2	3.9	12
C.L.O. 2	13.6	7.5	10

DISCUSSION

The maintenance of a normal concentration of calcium and of inorganic phosphorus in the serum is an example of biological regulation. The maintenance of a fairly constant body temperature, in the warm blooded animal, of a fixed hydrogen ion concentration in the blood, and of a constant osmolar concentration in the serum are other examples of such regulation. These phenomena involve the integration of biological and purely physico-chemical factors for the preservation of certain physiological equilibria. Various factors play a part in this regulatory mechanism. The concentration of calcium and phosphate in the diet, its content of vitamin D, as well as the concentration of other salts, the amounts of organic factors such as fat, carbohydrate and protein, and the reaction which the diet yields ultimately in the body or in the gastro-intestinal tract, all play a part in determining the absorption of bone forming salts. Endocrinological factors may determine the ebb and flow of calcium salts from and into the tissues or their elimination by the excretory organs.

Physico-chemical factors undoubtedly control in part the level at which calcium and inorganic phosphorus are maintained in the blood.

Absorption and Excretion of Calcium Salts

The literature dealing with the absorption and excretion of calcium salts has been critically reviewed by Stewart and Percival (19) and hence the interested reader will be referred to this article for details. The form in which the calcium is present in the diet in all probability affects its availability. Other facts may play a part. Only three variables have been studied. In these experiments the basal diet was kept constant and only the calcium, the total phosphorus and the fat soluble factor were varied.

In the absence of all but minimal amounts of vitamin D in the diet, physico-chemical factors seem to have full sway in the gastro-intestinal tract. Under these conditions insoluble or slightly soluble salts of calcium are less readily absorbed than soluble ones (20). At the reaction which prevails normally in the different parts of the small intestine of the rat; pH 5.2 to 6.5 (21), the less soluble phosphates of calcium, $CaHPO_4$ and $Ca_3(PO_4)_2$ as well as insoluble calcium soaps may be found in different proportions depending upon the pH of the intestinal contents, their content of phosphates, carbonates and fatty acids, the concentration of other salts, and possibly on other as yet undetermined factors.

With large amounts of Ca in the diet and minimal amounts of phosphorus, *i.e.*, high Ca/P ratio, much of the phosphate is precipitated as insoluble phosphate and is either very gradually absorbed or re-excreted in the feces without ever having actually entered the blood.[3] The excess of calcium is absorbed and produces a normal or even an excessive concentration of serum calcium. The failure of absorption of phosphate aided by the actual abstraction of phosphorus from the blood by the excess of Ca in the bowel, produces a lowering of the inorganic serum phosphorus. Although calcium was fed for the most part as $CaCO_3$, much of this was undoubtedly converted into chloride in the stomach while at the *p*H prevailing in the intestine it must have remained at least in part as $Ca(HCO_3)_2$, $CaHPO_4$, $Ca[H_2PO_4]_2$ and $Ca_3(PO_4)_2$, a small fraction remaining as $CaCO_3$ and calcium soaps. A similar mechanism may be responsible for the high inorganic serum phosphorus with low calcium and high

[3] One cannot conclude from the presence of calcium in the stools that ingested calcium has not been absorbed. Calcium may be absorbed and re-excreted into the intestine, when physical and chemical factors in the urine prevent its excretion by the kidney. That calcium derived from bone may find its way into the intestine is indicated by the excretion of amounts of calcium in the stools in excess of the amount ingested when animals and humans are fed a calcium poor diet.

phosphorus in the diet. After combining with Ca to form insoluble calcium phosphate the excess of HPO_4 and H_2PO_4 and their salts may remain. These are in turn absorbed and raise the inorganic serum phosphorus concentration above the normal level.

The work of Schabad (22) and of E. Schloss (23) has shown that the absorption of calcium phosphate is favored by the addition of cod liver oil. Even very small amounts of calcium in the diet suffice to maintain a normal concentration of this element in the serum. This holds true also for phosphate. The added fat, as such, does not account for this phenomenon. Vitamin D administered as ultra violet light (24) or irradiated ergosterol or as irradiated milk (25) acts in a similar manner. Not only may less calcium and phosphorus appear in the stools and more in the urine, indicating improved absorption of these elements, but more is retained. In fact the amounts retained are so large as to justify the conclusion that the excess is deposited in the bones, a conclusion which in the case of rachitic children can be verified by x-ray, or in experimental animals by analysis of the bones.

Cod liver oil, therefore, facilitates both the absorption and deposition of bone salts and in this manner regulates calcium and inorganic phosphorus metabolism. Vitamin D, the active principle concerned in this function, is more than an anti-rachitic factor since it exerts a controlling influence on calcium and phosphorus metabolism irrespective of whether rickets is present or not. Where the relative concentration of these elements in the diet is optimal for the animal so that normal concentrations of serum calcium and inorganic phosphorus prevail even with minimal amounts of vitamin D in the diet, the value of the cod liver oil addition is shown in the greater weight gain of the animals receiving the oil.

It is worthy of note that the minimal amounts of phosphorus absorbed under the influence of cod liver oil from low phosphorus, high calcium diets suffice to maintain a normal serum phosphorus level but are insufficient to allow normal gain in weight. There is not enough extra phosphorus for skeletal growth or the formation of protoplasm. Cod liver oil brings about the economical utilization of calcium and of phosphorus but it cannot wholly replace these substances; a certain minimum is essential.

At least two factors prevent the accumulation of calcium and phosphorus in the blood thus avoiding hypercalcaemia, hyperphosphataemia or both, namely the excretion of the excess by the kidneys or by the bowel or the deposition of these materials as insoluble calcium phosphate in the bones. The formation of insoluble calcium phosphate serves as a buffer preventing the excessive concentration of these elements in serum just as

the formation of weakly dissociated acids and their alkaline salts prevents the increase in the hydrogen ion concentration when strong acids are added to serum. Cod liver oil enhances the efficiency of the mechanism by facilitating absorption of calcium phosphate on the one hand and the deposition of the insoluble phosphate and carbonate of calcium on the other.

Cod liver oil increases the availability of both calcium and phosphorus and enables the body to function more economically with respect to these salts. The serum calcium concentration is restored to normal and excellent gain in weight is obtained even with minimal amounts of diet calcium. However, when diets containing minimal amounts of phosphorus are fed, the same amount of cod liver oil restores the serum inorganic phosphorus to the usual value but there is little growth. An increase of 0.2 per cent in food phosphorus increases the weight gain for the experimental period from 10 gm. to 77 gm. Further increase of diet phosphorus does not materially influence the concentration of inorganic phosphorus in the serum. A deleterious effect upon the weight is discernable from a large excess. Both serum calcium and inorganic phosphorus *values remain unaltered.*

But why should the normal concentration of calcium and of phosphate ion remain at a constant level in the serum, under normal conditions, and why must the level for the normal rat be approximately 10 mg. per cent of Ca = 2.5 mMol. of calcium and 8 mg. per cent of inorganic P or 2.6 mMol. of phosphorus? Undoubtedly physico-chemical factors determine this level. Calcium is present in serum as ionic calcium Ca^{++}, as Ca bound to non-diffusible ions, probably protein acids and as slightly dissociated but diffusible calcium salts. The relative proportion of these forms has only been determined approximately in the normal animal and varies in disease. Inorganic phosphorus at the pH of blood serum exists as $PO_4^{\equiv}$, $HPO_4^{=}$ and $H_2PO_4^{-}$. The equilibrium between these ions is dependent upon the pH of the serum, its total ionic strength, its temperature and probably other factors. It is possible that a dynamic equilibrium exists between the ions, calcium and phosphate, and the same ions in the tissue fluid bathing the bony trabeculae. There is some evidence that $CaHPO_4$ (25) plays an intermediary role in the deposition of calcium phosphate and recent, analytical and spectroscopic studies indicate that the inorganic matter of bone contains both $Ca_3(PO_4)_2$ and another calcium salt [Ca X]. Normal sera which have a calcium concentration of Ca = 2.5 mM and P = 5.0 mg. per cent or about 1.6 mM are in equilibrium with $CaHPO_4$. Higher concentrations are possible through the formation of diffusible or non-diffusi-

ble unionized but soluble compounds of calcium or phosphate. This explanation has been advanced by Greenwald (27) to explain the hypercalcaemia after overdosage with parathyroid extract and direct proof of the existence of soluble, non-diffusible weakly ionized, soluble compounds of calcium and citrate has been supplied by Shear and Kramer (28). Evidence for the clinical importance of such compounds has been supplied by Shelling and Maslow (29). The maintenance of a normal concentration of serum calcium and inorganic phosphorus is therefore the resultant of the rate of absorption of these elements from the gastro-intestinal tract, their rate of deposition in the tissues, particularly the bones, and the rate of excretion by the bowel and the kidneys and of certain physico-chemical conditions in the blood.

Conclusions

In these experiments the concentration of calcium and inorganic phosphorus in serum varied with the concentration of these components in the diet and with the amount of vitamin D. With minimal amounts of vitamin D the calcium of the serum varied directly as the calcium concentration in the diet. The same was true as regards the phosphorus of the serum. Increasing the calcium in the diet increased the calcium in the serum and depressed the phosphorus. The opposite effect was obtained when the phosphorus of the diet was increased. In the latter case, the calcium concentration was reduced to a minimal value unless an adequate amount of calcium was given to counteract this effect.

Deviations of the calcium and inorganic phosphorus of the serum from the normal, produced by marked disproportion in the concentration of these elements in the diet were less marked as the concentration of vitamin D in the diet increased. Vitamin D stabilizes the calcium and inorganic phosphorus concentrations in the serum. Both hypercalcaemia and hyperphosphataemia may be produced by appropriate dietary measures.

A Ca/P ratio in the diet of 1.5 (1.2 gm. of calcium and 0.8 gm. of phosphorus) gave the same value for the concentrations of these components in the serum, *i.e.*, normal values, irrespective of the amount of vitamin D. The effect of the vitamin D was evident in the superior gain in weight.

The calcium or phosphorus concentration of the diet, the Ca/P ratio, or the fat soluble calcifying factor may be the limiting factor in growth.

The regulatory influence of cod liver oil upon calcium and phosphorus metabolism has been confirmed.

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A TECHNIC FOR STUDYING LACTATION IN SMALL ANIMALS AND ITS USE IN EVALUATING PROTEIN LEVELS IN THE DIET

By

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IN ATTEMPTING to improve the current technic for studying the nutritional requirements of lactating rats, we have tested the three following possibilities: 1.—The prolongation of the lactation period of the mother by forcing her to adopt a series of new litters of animals, each younger than the preceding. This method was used previously in a few experiments by Maeder (1). This technic was abandoned after some preliminary trials due to the failure to get uniform results, the difficulty of getting all mother rats to adopt strange young litters and the complication of providing a regular supply of younger animals of the correct age. 2.—The feeding of the mother by mechanical devices that permit her to eat, leaving the young dependent solely upon the mother's milk before and after the weaning period. We have failed in this method thus far because the young rats have outwitted us in every attempt to develop a device that provides the mother with feed and excludes the young. 3.—The removal of the mother from the cage for separate feeding. This allows regular consumption of feed and regular periods for nursing the young. All the studies of this report were made with rats and employed this technic. Various feeding schedules were tried. The following proved satisfactory: 6 to 7 a.m., 9 to 10 a.m., 12 m. to 1 p.m., 3 to 4 p.m., 6 to 7 p.m., 9 to 10 p.m. Occasionally the last feeding at night was prolonged since the animal was more inclined to eat at this time. Among other schedules tested was one with an additional period from 3 to 5 a.m. This gave slightly better results but hardly justified the additional labor.

This technic which we have adopted rests upon the assumption that a relation exists between the food consumed by the animal and the milk secreted during a period of prolonged lactation. The advantage of this method is that it is possible to employ rats for lactation studies, to feed them synthetic diets and to put pressure upon the lactating mother for an optimum secretion of milk during a prolonged period. Thus we tend to exhaust effective stores of nutrients that may function for limited periods and give false results in short period studies. The chief disadvantages of

the method are the labor involved, and the large number of breeding females that must be withdrawn from the production of young in the stock colony. A third disadvantage that we have been unable to eliminate is the consumption of the mother's feces by the young. We have kept the litters upon false bottom cages with meshes sufficiently large to drop the feces, but some eating of them has been observed.

In order to get uniform results, we have used five mother rats with litters of the same age upon each experimental diet. To secure further uniformity we have provided litters of six rats for each mother by a suitable exchange of animals while quite young. In one series of experiments, Chart 2, the young of every mother were reduced to five per litter in order to determine if six animals consumed all the milk possible.

To test the suitability of the technic we have considered only one variable, namely the protein. The following are two typical diets we used:

	High protein	Low protein
Casein (commercial American)	40	10
Calcium carbonate	1	1
Bone meal	1	1
Sodium chloride	1	1
Lard	15	15
Yeast	5	5
Cod liver oil	5	5
Cooked starch	32	62

In order to insure an adequate supply of supplements, each mother was fed an additional half gram of dried yeast daily. Since we were primarily concerned in developing a technic, we have not worked with purified constituents. On the other hand we have worked with a diet composed of materials that lend themselves to limited purification in preference to such unknowns as dried meats or cereal grains. We have probably provided more yeast than necessary but wished to supply an adequate amount to prevent the B factors from introducing uncontrolled variables. In one series of experiments the young were fed yeast and cod liver oil separately with a medicine dropper, but since a limited trial did not alter the growth curves, this technic was not adopted.

In the preliminary trials it was found that the young maintained upon the milk of the mother grew very anemic. In the experiments reported this was prevented by administering small amounts of iron citrate, copper sulfate and potassium iodide to the young before they started to consume water. To each ten liters of water provided in the cages were added 3.5 gm. iron citrate, 0.1 gm. copper sulfate, and 0.008 gm. potassium iodide. This

afforded ample protection after the young started drinking. The young rats under these conditions did not become anemic.

In Chart 1 we have shown the mean food consumption of five mother rats upon the high protein diet and five upon the low level. We have also plotted the mean growth curves for the thirty young rats in each group. The curves are plotted upon semi-log paper and the probable error curves are shown on each side of the mean weight curve. The broken portion of the curves represents the periods when the young were dying. Photograph 1

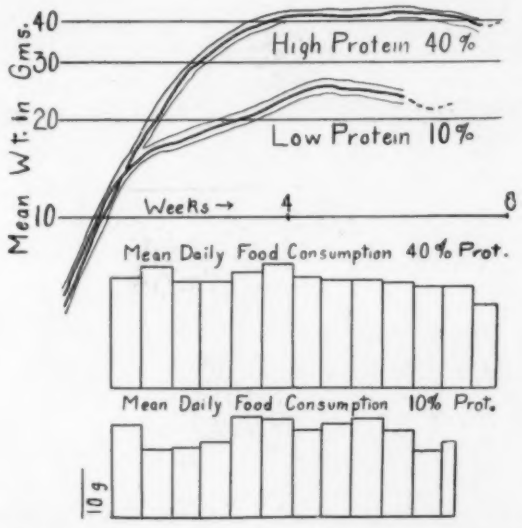


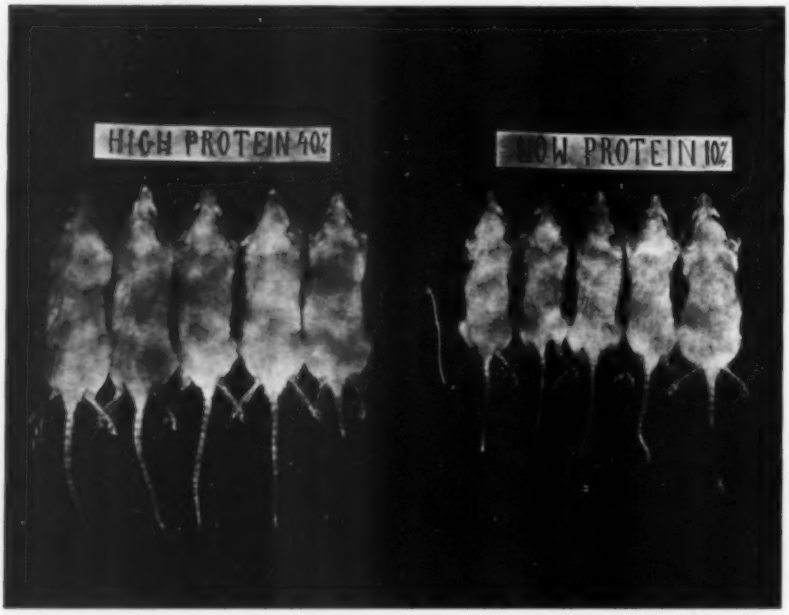
CHART 1.—Mean growth curves for thirty young rats nursed by mothers fed a high protein diet and for a similar number nursed by mothers upon a low protein intake. The food intake data represent the mean consumption per day per mother considered in time units of four days each.

shows the relative size of the rats just before the termination of the experiment. One typical young rat from each litter was selected for the picture.

In Chart 2 we have shown a similar experiment in which the protein levels were brought nearer together by using the same diets and exchanging casein for starch.

These data indicate that the technic is reliable in detecting the effect of different protein levels upon the secretion of milk. But the mothers upon the higher protein levels consumed more food. This contrasts with the observations of Adair (2) who found that nursing women upon high protein diets consumed less food. Since we felt the increased secretion of milk upon

the high protein diet might be merely the result of consuming more calories, we carried out a new series in which the mothers upon the high protein levels were limited in food intake to that consumed by those upon the lower levels. The result of this series is shown in Chart 3 and Photograph 2. From this last experiment one must conclude that the protein was responsible for the differences observed.



PHOTOGRAPH 1.—The relative size of rats nursed by mothers upon high and low protein diets.

The graphs show that lactating mother rats are unable to rear litters of six young much if any above the weight attained at the time of normal weaning. After attaining such a weight, say, about forty grams, they can be maintained upon the mother's milk until they are fifty or sixty days of age. At this time the milk supply seems to fail gradually. There is a considerable variation in mothers, however, and some litters were killed at the end of two months merely because other litters in the comparison had failed. Some mothers could have maintained their young for a much longer period.

The weight attained by the rats of Graph 2 is greater than that found in 1. This is due to the fact that only five animals were used in each litter

for the second experiment. We feel that this shows that six normal young rats take all the milk the mother secretes. When there are only five in a

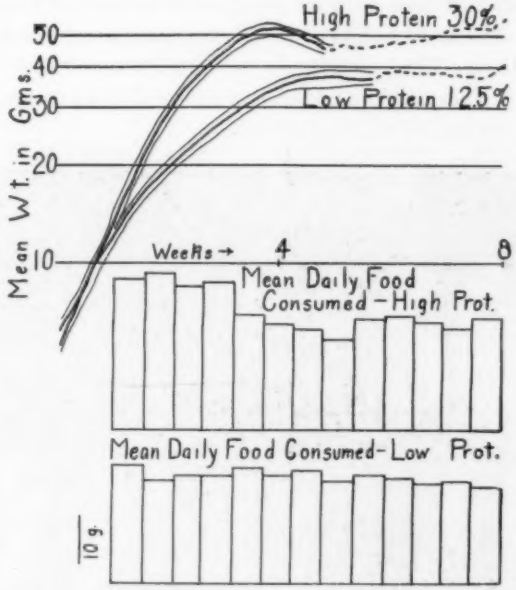


CHART 2.—Mean growth curves for nurslings when the number per litter is five instead of six and when the protein levels are close together.

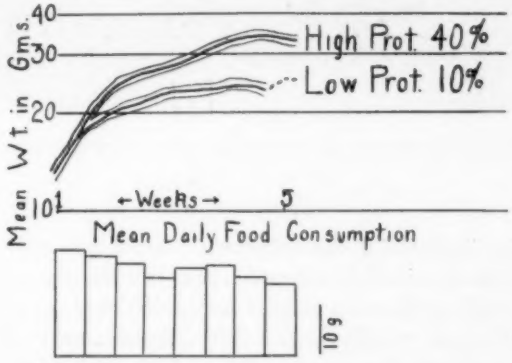
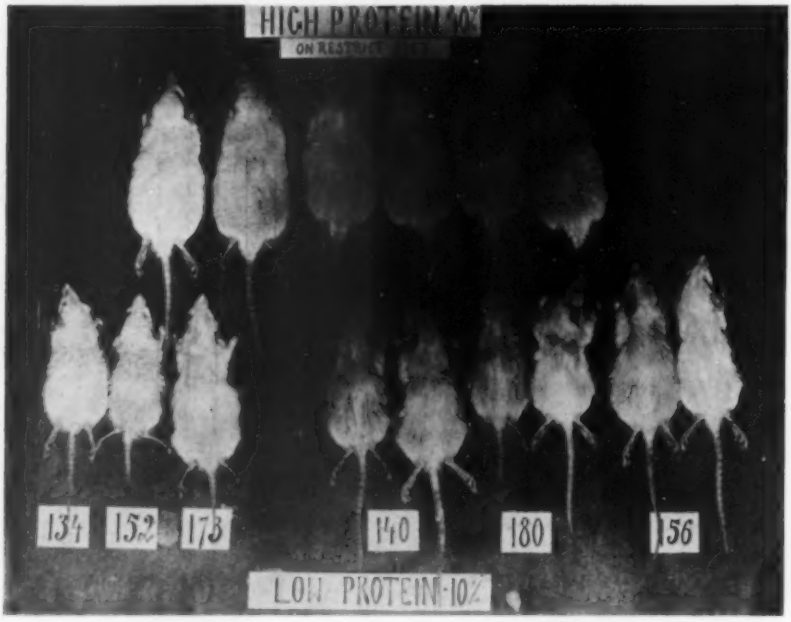


CHART 3.—Mean growth curve for thirty nurslings reared by five mothers upon high protein diets, contrasted with that for a similar group upon a low protein diet. The mean food intake per mother was the same in each group and was determined by the consumption of those upon the lower protein diet.

litter they attain a greater weight because there is more milk available per nursling.

Upon the high protein diets the mothers either gained weight or maintained their initial body weight, while at the lower levels there was a sacrifice of body tissue. These results do not conflict with those of Maynard and Bender (3) since casein alone cannot be considered a source of protein equal in value to several mixed proteins.



PHOTOGRAPH 2.—The relative size of rats nursed by mothers allowed equal intakes of high and low protein diets.

SUMMARY

A technic for determining the nutritional factors that control the secretion of milk in small animals has been devised. It is based upon the assumption that the prolongation of the lactation period may serve as a measure of nutritional factors. In this method litters are reduced to six in number. The nursing mother is allowed alternate periods for feeding and suckling her young. Anemia is prevented from developing in the young by feeding small amounts of iron, copper, and iodine before the animals begin to drink and by placing these elements in the drinking water when the young are

older. Under such conditions mothers can rear the young to about the weight of normal weaning and can maintain them until they are about two months old. With litters of five the weight attained is somewhat greater, indicating that six young consume all the milk secreted. There is a considerable individual variation among mothers. Some can extend their lactation period beyond the two months studied.

Casein fed at ten and forty per cent levels showed the lower one to be quite inferior. The nursing mothers consumed more food upon the higher protein level when they were allowed an unrestricted intake. In one series the intakes of the higher protein group were made the same as those upon the lower. The higher protein diet still produced superior results. Since much more work must be done before the answer to this protein problem can be given, we are presenting these experiments chiefly as an illustration of the technic.

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PROTEIN INTAKE AND BASAL METABOLISM OF COLLEGE WOMEN*

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ALTHOUGH a number of experiments have been carried on in an attempt to find out the protein intake and protein requirement of men, very little effort has been made to investigate the actual protein consumption and protein requirement of women. The present investigation was conducted on a group of healthy college women for the purpose of determining their habits of protein intake. Basal metabolism tests were also carried on and a study was made of a possible relationship between the quantity of protein ingested and the basal metabolic rate.

Sherman (1) in 1920 reported studies on the protein of the diets of adults who showed normal health and digestion. Using the total output of nitrogen as an indication of protein requirement, data were summarized from 109 experiments. Although 67 experiments were performed on men and 42 on women, only eight of the 37 subjects considered were women. The calculations showed that the average protein requirement for men or for women was practically identical when averaged to the same basis of body weight. The protein requirement was found to range from 21 to 65 grams with an average of 44.4 grams per 70 kilogram man per twenty-four hours. From these findings Sherman made the following statement concerning the protein needs in adult human nutrition, "A standard allowance of one gram per kilogram or one-half gram per pound per day appears to provide a margin of safety of 50 to 100 per cent as far as adult human nutrition is concerned." This standard allowance of protein supports the earlier view of Chittenden (2), who decided from his experiments on men and on dogs that the usual protein intake of men was far too high and that 60 grams or less of protein per day in the diet of a 70 kilogram man was ample for the nitrogenous needs of the body. In recent years reports have been made showing the nitrogen elimination of medical students. Brooks (3) in 1929 reported a study of the nitrogen excretion of 192 male medical students. Beard (4) in 1927 presented data for the nitrogen excretion of 400 male medical students. Denis and Borgstrom (5) in 1924 reported a study of a group of 242 medical students, including 9 women and 233 men. The nitrogen excretion per kilogram body weight was about the same for the women as for the men. The protein intake of individuals in these groups of medical students was shown to average slightly more than one gram of protein per kilogram body weight and thus satisfies the Sherman standard of protein consumption. The nitrogenous intake of these individuals is, however, far below that suggested by Pearl (6) in his report of 1920 and less than that recommended by Atwater (7) in 1895 and Benedict (8) in 1906. In a recent review of the question of protein intake, Mitchell (9) makes the following statement, "There are communities and races of men that have subsisted for generations on small amounts of protein

* Data for some of the experiments here reported were taken from the thesis submitted by Marie Killinger, in partial fulfillment of the requirements for the Degree of Master of Science in Home Economics in the Graduate School of the University of Illinois, 1929.

comparable to those advocated by Chittenden and Hindhede, but their physical, intellectual, and industrial characteristics are not such as to inspire confidence in the wisdom of their habits or any desire to follow in their footsteps." Mitchell concludes that with protein, as with other nutrients of the diet, it is possible to maintain nutritional health and physical efficiency over a wide range of intake.

In a series of observations made by the writer in relation to the protein intake of some of the Home Economics students at Iowa State College in 1927, it was found that the daily ingestion of protein was sometimes as high as 70 grams but more often it was as low as 25 to 40 grams and the average protein consumption of the group studied was somewhat less than the standard set by Sherman. Data for some of these observations were obtained from determinations of the excretion of nitrogen but more often the protein intake for this group of individuals was calculated from dietary records of food intake. The degree of accuracy of the data was thus uncertain but it appeared that the dietary habits of these college women were such that the protein intake was probably less than one gram per kilogram body weight. Further study of the amount of protein ingested by college women was carried on at the University of Illinois during the next three years. Determinations were made of the urinary nitrogen, basal metabolism, and protein and caloric intake of 85 women students. The majority of the subjects were juniors, seniors, or graduate students in the Home Economics department and most of them were interested in nutrition. These students lived the active routine of campus life and were healthy individuals whose weight approached normal and remained for the most part constant throughout the year. The group of individuals studied ranged in age from 19 to 37 years but 85 per cent of them were between 19 and 24 years of age. They weighed from 42.7 to 82.8 kilograms and the average weight for the group was 56.8 kilograms. The subjects offered their coöperation in this study and were extremely careful to record their food intake and to eat according to their usual habits during the days of the observations. The group studied included women who had their meals at sorority houses, at dormitories, at restaurants, and at home. The subjects recorded the kind and approximate amount of food eaten daily for at least one period of three days to a week. Very often the individuals served for two and sometimes three periods at different times of the year. An approximate estimate of the calories and protein of the diet was made and from the latter the nitrogen was calculated and compared with the total nitrogen excretion. At least two and usually three or more 24-hour urine specimens from each subject were analyzed for total nitrogen, creatinine, and acidity. A total of 320 twenty-four-hour urines from 85 women students

were analyzed. In some cases the analysis was made by the senior students but many of the determinations were carried on by Killinger.

Basal metabolism determinations were made on each of the 85 students of the group. These tests were made by Killinger or by Plant or by students whose work was under the direction of Plant. The Benedict-Roth metabolism apparatus was used and the per cent deviation of the actual basal metabolism from the normal prediction was calculated according to the Harris-Benedict standard. Three six-minute test periods were taken for each determination and at least two and sometimes three or more determina-

TABLE I

TOTAL NITROGEN EXCRETION IN THE URINE AND BASAL METABOLISM AVERAGED ACCORDING TO YEARS IN WHICH THE OBSERVATIONS WERE MADE

Year	No. of subjects	Average weights	Average urinary nitrogen per 24 hours	Average deviation of actual basal metabolism from Harris-Benedict prediction
		kg.	gm.	per cent
1928	21	59.5	7.81	−4.5
1929	27	55.1	8.66	−8.9
1930	37	56.6	6.84	−7.3
Average	85	56.8	7.69	−7.1
Average per 70 kg. man equivalent			9.48	

tions were made usually on successive days and always in the inter-menstrual period. The basal metabolism determinations were, as a rule, made on the same days upon which the 24-hour urines were collected. The tests were made before breakfast and in each case a one-half hour rest period preceded the determination. The oral temperature and the pulse rate were always taken.

In Table I are shown the averages of the results of the analyses of the 320 urine specimens. The averages of the results of the basal metabolism tests are also given. The urine analyses showed that the daily excretions ranged from 3.3 to 11.8 grams of nitrogen although the majority of the eliminations were between 6.4 and 8.9 grams. The average excretion for the group, as the table shows, was 7.69 grams of nitrogen. Since the average weight of the group was 56.8 kilograms, the equivalent 24-hour excretion for a 70 kilogram man would be 9.48 grams of nitrogen. Denis and Borg-

strom (5) at Tulane University found the average excretion of the group calculated for a 70 kilogram individual to be 11.07 grams nitrogen. Beard (4) at Western Reserve University found 11.16 grams nitrogen as the equivalent for a 70 kilogram man. And Brooks (3) at the University of North Carolina obtained 10.43 grams nitrogen as the equivalent 24-hour excretion for a 70 kilogram individual. These values are all higher than the 9.48 grams, the equivalent nitrogen excretion for a 70-kilogram person, calculated from our data on women students. When 10 per cent is added for protein loss through fecal nitrogen, the protein equivalent for a 70 kilo-

TABLE II

COMPARISON OF THE AVERAGE PROTEIN EQUIVALENT OF THE NITROGEN EXCRETION, AND OF THE BASAL HEAT PRODUCTION WITH THE ESTIMATED PROTEIN AND CALORIC INTAKE OF COLLEGE WOMEN

No. of subjects	Average weight	Average urinary nitrogen	Average protein equivalent of urinary nitrogen (adding 10 per cent for loss in feces)			Average total heat production per 24 hours (Benedict)	Food intake (estimated from diet records)		
							Average protein intake		Average caloric intake
			per kg. body wt.	per 56.8 kg. body wt.	per 70 kg. man equivalent		per kg. body wt.	per 56.8 kg. body wt.	
	kg.	gm.	gm.	gm.	gm.	cal.	gm.	gm.	cal.
85	56.8	7.69	0.94	53.4	65.8	1260	0.97	55	1700

gram individual, as shown in Table II, is 56.8 grams or 0.94 grams per kilogram body weight. The average protein intake for the women students is thus shown to be slightly less than one gram per kilogram body weight while the average protein intake of male medical students was shown by Denis and Borgstrom, by Beard, and by Brooks to be slightly more than one gram per kilogram body weight. It thus appears that the protein intake of the average college woman is probably somewhat lower per kilogram body weight than that of male medical students, and slightly below the Sherman standard.

The average basal metabolism of the 85 women in the group studied, as Table II shows, was 1260 calories per 24 hours. When this is calculated in terms of deviation from the normal, the average deviation of the actual basal metabolism from the Harris-Benedict prediction is, as Table I shows, −7.1 per cent. The deviations from standard for the various

individuals studied ranged from −20.0 to +11.0 per cent. Only 12 of the subjects showed a basal metabolism which was above the predicted value. All others showed metabolism values below the normal prediction. But of those whose metabolism was below the normal prediction only 17 showed an average deviation between the standard and −5.0 per cent. Thus even if, as Benedict (10) suggests, it is accepted that the standards are five per cent too high for women, over half of this group shows a metabolism below a standard which is lowered by five per cent, and the average metabolism (−7.1 per cent) of the entire group is lower than such a standard. Tilt (11) found for a group of young college women in Florida that the average deviation from the Harris-Benedict prediction was −9.9 per cent. She attributes this low value to the fact that the group studied by her is made up of southern women. Although our value is slightly higher than that reported by Tilt, there may not be sufficient difference to support a view that the basal metabolism differs for young women of the South and of the North. Our results do, however, show a wider range of variation, −20.0 to +11.0 per cent, than those reported by Tilt. Her results showed a variation which ranged from −18.5 to +1.8 per cent. Although the data presented in our investigation were obtained over a period of three years during the time from October to June, no difference in metabolism was observed with a change in season.

Since the group of women studied ingested on the average a fairly low protein intake and showed an average basal metabolic rate below the standard, it seemed possible that within the group an interrelationship might be apparent between protein intake and basal metabolism. The individuals of the group ingested daily quantities of protein which ranged from 0.5 gm. to 1.5 gms. per kilogram body weight and showed a range of deviation from normal basal metabolism of −20.0 to +11.0 per cent. In Table III summarized data are tabulated to show the basal metabolism averaged for groups ingesting different amounts of protein. The groups are, without doubt, too small to allow any definite conclusions. It seems apparent, however, from the data presented in Table III, that it is impossible to assign any definite relationship between habits of protein intake and basal metabolism. Although the small group whose daily intake was 0.5 gram protein per kilogram body weight showed the lowest basal metabolism, the group ingesting 1.2 grams protein per kilogram daily showed almost as low a basal metabolism. Also the average metabolic rate of the groups of individuals ingesting less than 0.9 grams protein is −6.5 per cent, a value slightly higher than the average for the entire group and about the same as −6.8 per cent, the average metabolism for the groups ingesting more

TABLE III

BASAL METABOLISM AVERAGED FOR GROUPS INGESTING DIFFERENT AMOUNTS OF PROTEIN

Individuals in different groups	Protein intake per kilogram per twenty-four hours	Average deviation of actual basal metabolism from Harris-Benedict prediction
Number	Grams	Per cent
6	0.5	−10.8
8	0.6	− 5.7
5	0.7	− 6.8
9	0.8	− 4.1
20	0.9	− 7.6
14	1.0	− 7.9
7	1.1	− 6.9
6	1.2	−10.4
5	1.3	− 4.9
4	1.4	− 4.4
1	1.5	− 4.0

than one gram protein per kilogram per day. Perhaps with a larger number of individuals and with data extending over a longer period of time, a more definite relationship could be demonstrated between protein intake and basal metabolism. Our present data appear to be in agreement with the recent findings of Wang and her associates (12) who could demonstrate no marked difference in the basal metabolic rate of subjects on different levels of protein intake. Kleitman (13), Deuel and his associates (14), and Wishart (15), on the other hand, observed a very definite difference in basal metabolic rate on different levels of protein intake. They found that the basal metabolic rate decreased as the protein intake fell and increased when the subject was put upon a high protein diet. In view of the findings of these investigators it seems possible that the somewhat lower protein intake of women may be in part responsible for the fact that their basal metabolism is usually lower than that of men.

SUMMARY

A study of the urinary nitrogen excretion, basal metabolism, and food intake of 85 college women was conducted for the purpose of determining the habits of protein consumption in women and the possible interrelationship between protein intake and basal metabolic rate.

The average daily protein intake of the group studied was 0.94 grams per kilogram body weight, a value slightly lower than the daily protein in-

gestion characteristic of male medical students as determined by Denis and Borgstrom, by Beard, and by Brooks.

The average twenty-four hour urinary nitrogen excretion for the group was 7.69 grams.

The average basal metabolism for the group was found to be lower than the standard. The deviation of the actual basal metabolism from the Harris-Benedict standard averaged −7.1 per cent.

There appeared to be no definite interrelationship, within the group, between protein intake and basal metabolic rate. It is suggested that the lower protein intake of women may be in part responsible for the fact that their basal metabolic rate is usually lower than that of men.

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VARIATIONS IN BLOOD SUGAR VALUES OF NORMAL AND VAGOTOMIZED DOGS FOLLOWING GLUCOSE ADMINISTRATION

By

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WE HAVE attempted to establish a method by which the administration of glucose would afford reliable information regarding the ability of an animal to burn or store (metabolize) sugar.

For this investigation we have employed the dog, an animal whose blood sugar level may remain relatively constant over a period of several hours. Quiet, gentle animals were selected and as a result of extensive training they would lie completely relaxed (although unanesthetized) for a period of hours on the beds provided. It was especially noted that they gave no evidence of psychic disturbance as the result of experimental manipulations, e.g., handling or the collection of blood samples. To reduce errors related to the absorption of previously administered food, the animals were in a fasting condition at the time of the experiment. The determinations were therefore made with the subjects in the basal condition. Errors related to variations in the rate of movement of material through the gut, and also the rate of absorption, were avoided by administering the glucose intravenously. We employed an amount of glucose which we determined by preliminary experiments was approximately the maximum which could be given under these conditions without losing glucose by way of the kidney. We made determinations of "true" blood sugar values; the method of Somogyi (1) was employed for these determinations.

Normal dogs, fasting 40 hours, received intravenously 0.25 gm. per kg. of glucose in the form of a 50 per cent solution. Approximately one minute was required for this injection. Blood samples were taken just preceding the injection and also 15, 45, 90, 180, 270 and 320 minutes after the injection.

A decrease in the demands made on the Islet cells of the pancreas, such as occurs during fasting, is believed by certain observers to reduce the readiness with which insulin is made available when glucose is subsequently administered. To test this theory, the experiment was repeated with dogs which had not been fed for 40 hours but to which 25 grams of

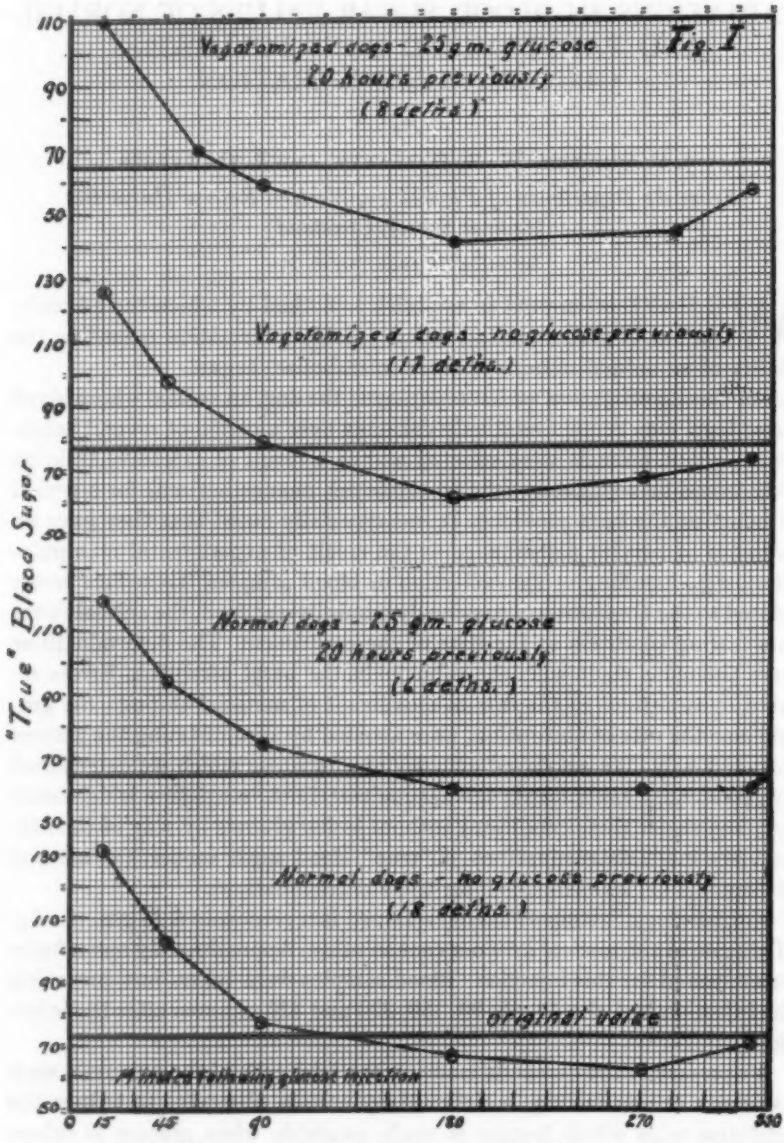

FIG. 1.—"True" blood sugar values following intravenous injection at 0 minutes of 0.25 gm. glucose per kg. Dogs fasting 40 hours.

glucose by stomach tube had been given 20 hours preceding the intravenous administration of the test solution of glucose.

Recent experiments of La Barre (2) have definitely shown that the vagi can be of great importance in determining the blood sugar level (or rate at which insulin is made available) following the administration of glucose. Since dogs with chronic bilateral vagotomy grossly show no indications of insulin insufficiency, we have employed the methods described above to compare such animals with normal dogs. We have not concerned ourselves with the acute changes which might follow section of the vagi (made just above the diaphragm) but have used animals 1 to 6 months after operation, animals in which adaptive changes would be well stabilized.

As was to have been anticipated, the blood sugar levels of animals apparently under the same experimental conditions showed some fluctuation. Certain animals gave figures consistently higher than others throughout the investigation and the curves obtained with a group of animals one day frequently were in closer agreement with each other than with those obtained from the same group on a subsequent day. The results of our investigation can most satisfactorily be presented in the form of graphs (Fig. 1).

Examination of these curves shows that the true blood sugar level of normal dogs fasting 40 hours averaged 73 mgm. It was practically the same in the vagotomized animal but decidedly lower in both groups of animals if fasting 40 hours and given 25 gm. of glucose 20 hours preceding the experiment. Fifteen minutes following the intravenous injection of glucose, the blood sugar level was lower in both groups of animals if dextrose was administered orally 20 hours previously. The blood sugar value regained the original level earlier in vagotomized animals and the fall below the original (degree of undershoot) was more marked than in the normal animal.

SUMMARY

A method is proposed by which a determination of the rate at which glucose is removed from the circulation following the administration of glucose would give information regarding the ability of the animal to metabolize sugar.

This method has been employed on normal and on double vagotomized dogs. Curves showing composite results are presented.

If glucose is administered 20 hours preceeding the test, an increase in the ability to metabolize sugar is noted in vagotomized but not in normal animals.

Chronic vagotomy does not reduce the sensitivity of the mechanism which is stimulated by the administration of glucose; the mechanism rather appears to be slightly more irritable.

If these results are to be interpreted as indicating the rate of insulin secretion, they tend to show that hyperglycemia will lead to at least as great a secretion of insulin in the pancreas freed from central vagus control as in the normal Islet tissue.

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Editorial Review*

EGG-YOLK PROTEINS

1. Introduction

FOR many centuries, long before it was shown to contain not only readily-digestible protein of high biological importance but also iron, phosphorus, calcium and vitamins A, B, D, E and G, the egg has been recognized as one of the most valuable constituents of human diet. Particularly is this true of the yolk of egg, which, apart from its deficiency of carbohydrate and of the antiscorbutic factor, appears to contain all the food substances required for the normal development of young mammals. It is therefore all the more astonishing to realize, in spite of the large amount of time which, has during the past twenty years, been devoted to the study of proteins from the academic point of view, of egg production from the more technical standpoint, and of food constituents from both these aspects, that our knowledge of the chemical components of the egg-yolk has advanced so slowly.

We ourselves, engaged at present in a chemical investigation of the yolk proteins, venture to hope that it may prove useful for others interested to have before them a short review of the principal work which has already been done in this field. In this contribution we intend to limit ourselves, in the main, to a discussion of the chemistry of the yolk proteins of the unincubated egg. Work on the metabolism of the developing egg has already been adequately summarized up to 1924 by Needham (1) and more recent results of Calvery (2) have supplemented Needham's review. In order to keep the present article within reasonable and readable dimensions, we are excluding, as far as possible, the consideration of the other constituents of egg-yolk, namely, fats, lipins, sterols, pigments, vitamins, and salts.

In a brief review of this nature there must almost inevitably be errors of omission, and it may even be that some really important contributions have been overlooked. We hope that such omissions are few but would apologize in advance to any authors whose work we have unwittingly neglected.

2. The Isolation of Egg-Yolk Proteins

(a) *Early work.* If its value is necessarily limited by the primitive conceptions of protein chemistry then extant, there is a certain historical interest in the work of the eighteenth and early

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nineteenth century investigators on the yolk of egg. The earliest mention we have been able to find of protein in the yolk is that of Fourcroy (3) in 1782, who stated that yolk is chiefly "albumin," fat being the substance second in importance. Sixty years later, in 1841, Bence-Jones (4) coagulated egg yolk by heat and extracted fat from the coagulum with ether. On analysis of the residue its elementary composition agreed well with the usually accepted values for protein. Dumas and Cahours (5), a year later, named this protein "vitellin." Lehmann and Messerschmidt (6) in 1842 were the first to call attention to the unusual physical properties of this compound. They demonstrated that the precipitate which forms when egg yolk is mixed with water is easily soluble in either sodium or ammonium chloride solution, is reprecipitated from such solution by adding excess of water, and may be taken up again in salt solutions. Denis (7) made a further contribution to the method of purification of vitellin. After straining egg yolks through linen, he extracted them with ether until the extracts were color-free. The fat-free vitellin he found to be insoluble in water and saturated NaCl, but readily soluble in dilute acids, alkalies and 2 per cent and 10 per cent NaCl. It was rendered insoluble by long contact with water.

Apparently the first investigators to recognize the existence of two proteins in the egg yolk were Valenciennes and Frémy (8). They state that vitellin in the bird's egg yolk is always found associated with a certain quantity of albumin, hence, to prepare vitellin, they treated the egg yolk of the hen with cold water, the albumin remaining in solution while the vitellin was precipitated. The latter, washed with water, alcohol and ether, they regarded as a very pure protein.

An advance in the knowledge of the chemistry of vitellin was made by Hoppe-Seyler (9) who stated that vitellin was a lecithoprotein and showed that the lecithin could be split off by boiling alcohol (10).

Kossel (11) found that although the compounds guanine, hypoxanthine, and adenine could be separated from the "nucleins" of 15-day chick embryos, all of these compounds were absent from the yolk of unincubated eggs. He pointed out that the "nuclein" of milk (casein) and that of egg yolk (vitellin) contained no purine bases and were therefore essentially different from the "nuclein" (nucleoproteins) of the cell. In spite of this important observation, many years elapsed before a clear differentiation in nomenclature was made between nucleoproteins and phosphoproteins.

(b) *Work since 1899.* It is to be noted that the definition of vitellin as made originally by Dumas and Cahours had undergone modification. The name was now applied to the purified product prepared on the lines suggested by Denis' (7) work. Vitellin, as defined in this way, was first prepared in a reasonably pure form in 1899 by Osborne and Campbell (12). They mixed egg yolk with an equal volume of saturated NaCl, extracted the mixture repeatedly with ether, and dialyzed the fat-free residue. They obtained a series of fractions of various solubilities, which were found to contain from 15 to 30 per cent of "lecithin" on alcoholic extraction, the more soluble fractions containing the larger proportion of lecithin. It was found, however, that the insoluble residue obtained after a thorough extraction of any of these fractions with boiling alcohol had a very nearly constant composition (see Table I, substance 6).

In the same year Gross (13) extracted the ether-soluble materials from egg yolk, and filtered the resulting fat-free, whitish suspension through a Chamberland filter candle. Based partly on the investigation of the products obtained by this method of separation, and partly on an examination

TABLE I

PERCENTAGE COMPOSITION OF THE PROTEINS OF THE YOLK OF THE HEN'S EGG

	Vitellin 1	Vitellin 2	Vitellin 3	Vitellin 4	Vitellin 5	Vitellin 6	Vitellin 7	Livetin 8	Vitellin 8
Carbon					52.71	51.24			
Hydrogen					7.46	7.16			
Oxygen					22.14	23.24			
Nitrogen					16.64	16.38		15.35	
Sulphur					1.05	1.04		1.80	0.6
Phosphorus						0.94		0.067	
Glycine	1.10	0.00	trace	0.5					
Alanine	+	0.75	0.16	0.5					
Valine	2.40	1.87		1.5					
Leucine	11.00	9.87	3.30	6.8					
Proline	3.30	4.18	4.00	0.5					
Phenylalanine	2.80	2.54	1.00	0.7					
Aspartic acid	0.50	2.13	0.6	0.7					
Glutamic acid	12.20	12.95	1.00	1.0					
Serine	?	?		0.5					
Tyrosine	1.60	3.37	0.40	2.0				5.2	5.0
Cystine		?		—				3.9	1.4
Histidine		1.90	trace	2.1			1.4		
Arginine		7.46	1.20	1.0			7.6		
Lysine		4.81	2.40	1.2			6.0		
Ammonia		1.25		1.17					
Tryptophan		+						2.1	1.6

1. Abderhalden and Hunter (18).
2. Osborne and Jones (21).
3. Levene and Alsberg (20).
4. Hugounenq (19).
5. Osborne and Campbell (12).
6. Same as (5), but calculated to include the phosphorus.
7. Plimmer and Rosedale (22). Used the total egg-yolk protein.
8. Kay and Marshall (15).

of the fractions obtained by ammonium sulfate precipitation, the conclusion was arrived at that a second protein, a globulin, was present in egg yolk. Credit for the recognition of the existence of this second protein belongs, as has just been stated, to Valenciennes and Frémy, and although Gross' work supported their results, a careful examination of his original paper leads us to the conclusion that at no time did he get a clear separation of the globulin and the vitellin. This was left for Plimmer (14) to achieve. Vitellin was prepared by diluting an ether-extracted solution of egg yolk in 10 per cent sodium chloride with a large volume of water, and filter-

ing off the precipitated protein. There was still a relatively large amount of protein nitrogen in the filtrate. To the filtrate, therefore, was added a little acetic acid, and the mixture boiled. The soluble protein was thus coagulated and could be filtered off and washed. Plimmer gave the name "livetin" (an anagram of vitellin) to this coagulum of which he obtained several grams. His yields varied from 10 gms. from 32 egg yolks to 30 gms. from 25 egg yolks. On analysis he found the phosphorus content to vary from 0.1 per cent to 0.65 per cent in 5 different specimens, being in all cases markedly lower than that of vitellin (1.0 per cent or above). He determined the nitrogen partition by the Hausmann method and found that the figures agreed fairly closely with those obtained for vitellin, but differed from those for ovalbumin. Livetin was higher in monoamino-N than vitellin, but lower in amide-N and diamino-N. He put forward the suggestion that the product he separated might be closely related to vitellin, might be, in fact, vitellin minus the phosphorus-containing fraction of the molecule. Although Plimmer brought evidence to show that his product was not ovalbumin, he did not demonstrate clearly that the proteins of the white did not contribute to his coagulum.

After Plimmer's work, nothing appears to have been done on the second protein of egg yolk until 1928, when Kay and Marshall (15) first obtained it in a fairly pure, undenatured form. After freeing the yolks completely from adherent proteins of the white, they were taken up in sodium chloride solution and extracted with ether. The fat-free protein solution was dialyzed against running water for a short time, and then poured into distilled water, until no more precipitate formed on further dilution. Most of the lecitho-vitellin was centrifuged off, and the remainder removed by filtration. The livetin was then precipitated from the filtrate by half saturation with ammonium sulfate and after repeating this precipitation twice more the product was extracted with alcohol-ether at $-15°$ by Hewitt's (87) method, which effectively removed traces of lipins remaining in the livetin. The resulting purified but uncoagulated protein had the properties of a pseudo-globulin, and on analysis was found to contain 15.1 to 15.35 per cent N, 1.8 per cent S, and 0.067 to 0.05 per cent P, the purer specimens having the smaller content of phosphorus. Amino-acid analysis by the methods of Folin and Looney (88) and Folin and Ciocalteu (89) gave 5.2 per cent tyrosine, 2.1 per cent tryptophane, 3.9 per cent cystine, whilst pure vitellin analyzed by the same methods at the same time gave 5.0 per cent tyrosine, 1.6 per cent tryptophane and only 1.4 per cent cystine, with a total sulfur content of 0.6 per cent. Plimmer's suggestion of the possi-

bility of a direct relationship between vitellin and livetin is not borne out by these results (see Table I).

Thus two definite proteins have been isolated from the yolk of the hen's egg, the first, a phosphoprotein, vitellin, which when combined with lecithin has globulin-like properties, the second, livetin, of very small phosphorus content and with the properties of a pseudoglobulin.

3. Chemical Characterisation of the Yolk Proteins

Plimmer and Scott (17) showed that phosphoproteins such as casein and vitellin could be distinguished from nucleoproteins by the stability of the combined phosphorus of the latter to 1 per cent sodium hydroxide. The phosphorus of phosphoprotein was readily separated as inorganic phosphate by this treatment. Plimmer and Scott showed that the phosphorus-containing proteins of the roes of the sturgeon, herring, and mullet were similar to casein and vitellin in this regard.

Following the introduction by Fischer of the esterification method for determining amino-acid distribution, there was great activity for some years in the chemical investigation of proteins. The amino-acid content of vitellin was determined by Abderhalden and Hunter (18) who do not state their method of preparation; by Hugounenq (19) who used the heat-coagulated, total egg-yolk protein; by Levene and Alsberg (20) whose preparation, judged from the method given, must have been free from livetin; and by Osborne and Jones (21) who used a product which also must have been free from livetin. Their analytical results, and those of other workers on vitellin and livetin, are given in Table I.

Vitellin was found to contain 1.74 per cent tryptophan by May and Rose (23), 1.1 per cent tryptophan by Tillmans and Alt (24), 2.42 per cent tryptophan and 0.83 per cent cystine by Jones *et al.* (85). Ide (86) stated that dried egg yolk contained 2.46 per cent tryptophan. Livetin was found to contain from 14.82 to 15.35 per cent N, and 0.10 to 0.85 P by Plimmer (14). Other chemical properties of the yolk proteins are dealt with in later sections.

4. Physical Properties of the Yolk Proteins

Vitellin. Purified lecitho-vitellin is insoluble in water, and requires a rather greater concentration of salt to take it up into solution than does the typical globulin. The amount required varies with the length of time during which the protein has been in contact with water. After thorough extraction with ether of the yolk diluted with an equal volume of 8 or 10

per cent sodium chloride solution, and precipitation of the vitellin with water, there is still a considerable amount of "lecithin" loosely combined with the protein. This may constitute as much as 30 per cent or even more of the dry weight of the protein. At this stage the vitellin, or better, lecithovitellin, is easily soluble in 1 per cent salt solution or in dilute alkali, from which it can readily be precipitated by acidification. On keeping in contact with water, "lecithin" is slowly lost by the protein, which at the same time becomes less and less soluble in salt solution. After complete removal of the lipin, which requires treatment of the protein with hot ethyl or methyl alcohol, the vitellin becomes quite insoluble in water and almost insoluble in salt solutions or dilute alkali. It is, however, readily decomposed after suspending in dilute alkali, even at room temperature, both phosphoric acid and ammonia being liberated from organic combination.

Since vitellin becomes progressively less soluble in water the more it is purified, it is not surprising that few determinations of its physical constants have been attempted.

Livetin. Livetin, though completely precipitated by 50 per cent saturation with ammonium sulfate, is nevertheless soluble in water, or rather requires such small quantities of salt for solution that even on prolonged dialysis it remains for the most part unprecipitated. It may be said to have the properties of a pseudoglobulin. It is precipitated from aqueous solution by complete saturation with magnesium sulfate or sodium chloride, by three volumes of alcohol or acetone, by trichloracetic, picric, tungstic, and sulfo-salicylic acids, by the salts of the heavy metals and by colloidal ferric hydroxide (15). Certain preliminary determinations of its physical constants have been made (15). (a) *Its isoelectric zone* in acetate buffers, is in the neighborhood of pH 4.8–5.0, and its titration curve shows a broad isoelectric zone in this region; (b) *The refractive index* for 1 per cent protein gives a mean value of 0.00190 at 20°C; (c) *The optical rotatory power* for the $\frac{7}{1}$ mercury green line determined on pure, alcohol-ether extracted specimens gives a mean value for the specific rotation at 20° as follows: $[\alpha]^{20°}_{5461} = -55.5°$. (d) *The minimum molecular weight*, on the assumption that there is only one atom of P in each molecule of protein, is in the neighborhood of 64,000.

Relative quantities of vitellin and livetin present in the yolk. A method for estimating the amount of livetin in the yolk of a single egg was developed by Kay and Marshall (15) on the assumption that all the non-vitellin protein in egg yolk is the same substance—livetin. (They considered at least 90 per cent of the non-vitellin protein to be livetin.) By this method

they found, both for hen's and duck's eggs, the ratio of vitellin to livetin to be between 3.3:1 and 4:1, i.e., between one-quarter and one-fifth of the yolk proteins of the hen's or duck's egg is livetin—the rest being vitellin. On this basis, and the figures given for sulphur content of yolk proteins in Table I, a short calculation will show that about one-half of the sulfur of the yolk is originally present as livetin. Whether their assumption (that at least 90 per cent of the non-vitellin protein in egg yolk is one protein—livetin) is entirely justified still remains to be seen. So far there is no definite evidence against it, except that yields of purified livetin are lower than might be expected on this assumption.

The ratio of vitellin to livetin in the egg yolk in the few cases in which it has been determined appears to be fairly constant. Whether this ratio varies in any way with the diet has not been ascertained. One possible explanation of the constancy of the ratio is that the two apparently distinct and certainly artificially-separable proteins are secreted or manufactured as one, and maintained in the natural state in the egg yolk as one large molecule which is decomposed at some stage during the process of fat extraction and "separation." If this is the case, the strength of the affinity between the two portions of the molecule must be small—considerably smaller, for example, than that between vitellin and lecithin in lecitho-vitellin. This question, however, has considerable interest in considering the mode of formation of the yolk, and may be of importance in yolk utilization by the growing embryo. Whether the two proteins are digested and absorbed by the embryo as one, or at least at the same rate, has not yet been determined.

There is, however, another reason why it is unlikely that the two proteins are in a state of loose chemical combination in the yolk. In some recent experiments (108) we have obtained evidence which suggests that livetin is very closely related to hen's serum globulin, and may, in fact, be identical with it. If these preliminary findings are confirmed, they would suggest a different origin[1] for the livetin and for the lecitho-vitellin of egg-

[1] A very short summary of some of the work on the minute anatomy of the formation of egg yolk and the yolk proteins may be given here.

The vitelline membrane enclosing the yolk is something more than a mere receptacle. Lecaillon (102) found that in the bird *Turdus merula* the membrane was associated with an internal layer, 3μ in thickness, to which adhered fragments of the follicular epithelium and of the connective tissue of the theca from the ovary. Thing (103) observed in the ovarian egg of the turtle that prolongations of the epithelial cells of the yolk membrane traversed the zona pellucida and ended in knob-like enlargements in the yolk substance itself, presenting a very favorable arrangement for the conveyance of nutritive material from the epithelial area, in contact with the maternal capillaries, to the actively growing and extending yolk.

The laying down of yolk material, both protein and non-protein, within the growing egg yolk

yolk—the latter then being a specific protein constituent manufactured by the ovary, the former merely a translocated blood constituent. It is suggestive in this connection that Horvath (107) found recently that out of a number of laying hens whose blood constituents were determined, and all of which had a relatively high globulin to albumin ratio in the serum, the two best layers had by far the highest serum globulin figures (4.3 and 4.96 gms. per 100 cc. serum).

5. Yolk Proteins in Non-Avian Eggs

It is of considerable interest to compare the results just described for the proteins of the yolks of avian eggs with those which have been obtained for the yolk proteins of the eggs of other classes of vertebrates.

Gobley (25), writing in 1850, states that Vauquelin in 1817 analyzed the eggs of the pike, and found much albumin, oily material, a gelatin-like substance, and mineral salts. Gobley himself used carp eggs and found them to contain 14 per cent of "paravitellin," a protein which he obtained by triturating the eggs with distilled water. He found iron present in the eggs in a fat-soluble form, and describes at some length his excellent and well-ordered researches upon the various fractions obtained from the eggs.

Valenciennes and Frémy (8) examined the eggs of many species of fish. From the eggs of cartilaginous fishes (principally rays and dogfishes) they isolated "ichtin" in which could be found no sulfur, while from the eggs of bony fishes they obtained "ichthulin," analogous to vitellin, containing 1.0 per cent S.

Walter (26), in 1891, prepared and purified ichthulin from carp eggs. His investigation is the only one we have been able to discover in which the iron content of this protein is recorded. Two determinations, by different methods, gave him 0.09 per cent and 0.117 per cent—rather high values. Ten years later Levene (27) extracted ichthulin from cod-roe by repeated solution in 5 per cent NH_4Cl and precipitation by dilution. He noted that the substance became orange-yellow after extraction with absolute alcohol and ether.

Hugounenq (28) isolated a water-insoluble protein from the eggs of the herring, which he named "clupeovin." He regarded it as a complex, feebly acid protein very similar to the vitellin of birds' eggs. It was readily soluble in dilute alkalies and in dilute HCl, and contained small quantities of P and Fe. A similar protein was obtained by Galimard (29) from the eggs of *Rana esculenta*, and named "ranovin." Ichthulin from the eggs of *Squalus acanthias* was prepared by

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is a process the understanding of which is of first importance if the biochemical relationship of the hen to the egg is to be made clear. What concerns us more immediately at present is the production of the yolk protein by the hen, but this cannot, as yet, be disentangled from the production of the other yolk constituents. That this process is concerned with the so-called "vitelline body of Balbiani," which is to be found in the cytoplasm of the young ovules of a wide variety of species, seems to be the opinion of several cytologists. Henneguy (104) in 1893 described this structure as a finely granular mass situated not far from the periphery of the egg, with a small, more deeply staining body centrally within it. D'Hollander (105) studied the cells in the ovary of the embryo chick, and showed that Balbiani's body is already present in the oöcyte. It is closely related to the attraction sphere—the dormant centrosome. He considered that the body of Balbiani, surrounded by its "vitellogenic bed" played a major part, especially in birds, in the formation of the nutritive yolk. Lams (106) working with the frog *Rana temporaria* stated that during the growth of the oöcyte, its cytoplasm is increased in quantity by "deutoplasmic bodies of a mitochondrial nature" elaborated under the control of the sphere of attraction, which remains in the oöcyte as the vitelline body of Balbiani.

Alsberg and Clark (30). After ether and hot alcohol extraction they found, rather surprisingly, that phosphorus was absent, and that only slight traces of iron could be detected. Attempts to produce hematogen (see p. 91) from it by the usual methods successful with vitellin, yielded very small amounts of a substance which contained no P and but slightly more Fe than the original material.

Reinvestigation of this dogfish egg-yolk protein has been made recently by Needham (31). He was able to show that, like avian egg-yolk protein, it could be separated into two fractions, the ichthulin fraction, analogous to vitellin, and another fraction, similar in properties to livetin, which he called "thuichthin." The phosphorus content of the former protein after thorough extraction with boiling alcohol he found to be 0.62 per cent, which is not in agreement with the findings of Alsberg and Clark, whilst "thuichthin" contained only 0.039 per cent of phosphorus.

Fauré-Frémiet and Garrault (36) found the ovarian egg of the carp (*Cyprinus carpo*) to contain 25.7 per cent of its total weight as protein. They extracted it with boiling alcohol and estimated its nitrogen and phosphorus content. They also (37) report similar results with a protein from the eggs of the trout (*Trutta fario*).

Steudel and co-workers (34) give figures for the basic-N distribution of herring ichthulin when analyzed according to the method of Kossel and Kutscher (90).

McClendon (32) found the yolk platelets of the egg of *Rana pipiens* to contain 94 per cent of a protein which he named "batrachiolin." Its composition and precipitation reactions indicated its relationship to vitellin and ichthulin. Later (33) he found that the protein dissolved slowly in salt solutions, but was more readily soluble in alkalies. It was completely precipitated by half saturation with ammonium sulfate. The black pigment of the eggs he found to contain 0.483 per cent P, 0.83 per cent S, and 10.9 per cent N; he states, however, that the phosphorus content of the pigment was apparently due to contamination with batrachiolin.

Fauré-Frémiet and de Stréel (35) separated "vitellin flakes" from the eggs of *Rana temporaria* by centrifuging the crushed eggs. They were able to separate partially two protein fractions by treatment with N/10 NaOH, the soluble part (37%) being a nitrogen compound rich in P; the residue was a nitrogen compound containing little or no P but some S, and very resistant to reagents.

Fauré-Frémiet (38) separated "vitelline globules" from the eggs of a marine worm, *Sabellaria alveolata*, and found them to contain two nitrogenous fractions, one apparently an albuminoid, the other soluble in dilute alkali and containing 0.51 per cent of P.

Komori (57) prepared a protein from the spawn of the gastropod *Hemifus tuba* (Gmel) by extracting with alcohol and ether the coagulum obtained by boiling the spawn in dilute HCl. He quotes for the amino-acid content of this "crude vitellin," glycocoll 0, alanine 0.71 per cent, valine 0.27 per cent, leucine 10.29 per cent, isoleucine 0, proline 1.10 per cent, phenylalanine 0.22 per cent, aspartic acid 1.60 per cent, glutamic acid 0, serine 0, tyrosine 0.80 per cent, arginine 3.73 per cent, histidine 0, lysine 0.86 per cent, tryptophan 1.49 per cent.

Tomita (39) found the eggs of the sea turtle (*Chelonia cavana*) to contain 39 per cent of white, of which only 1.5 per cent was solid matter, and 55 per cent yolk. Thirty-seven per cent of the yolk was solid matter, containing 10.7 per cent N and 4.3 per cent ash.

The analytical findings of several of the above mentioned investigators are given in Table II.

6. White and Yellow Yolk

The question of the homogeneity or otherwise of the egg yolk has not yet been discussed. It is not difficult to show, in a hard-boiled egg, that the yolk is made up of alternate layers of white and yellow yolk. That the difference between these layers is more deep seated than one of mere pigmentation has been shown by the work of Riddle, and of Riddle and Spohr.

TABLE II

PERCENTAGE COMPOSITION OF THE PROTEINS OF NON-AVIAN EGG YOLK

	Paravitellin 1	Ichthulin 2	Ichthin 3	Ichthulin 4	Clupeovin 5	Ranovin 6	Ichthulin 7	Ichthulin 8	Ichthulin 9	Thuichthin 9	Ichthulin 10
Carbon	52.6	53.52	51.0	52.5	53.68	53.61		50.49	48.80	45.60	52.44
Hydrogen	7.74	7.70	6.7	8.0	7.38	7.79		7.16	7.58	7.39	7.45
Oxygen	25.24	22.19	25.4	22.7	23.90	24.27					22.58
Nitrogen	15.15	15.64	15.0	15.2	14.64	15.32		16.76	14.75	12.14	15.96
Sulphur	0.90	0.41	—	1.0	0.40	trace		0.91	1.17	1.96	0.92
Phosphorus	0.37	0.43	1.9	0.6	trace			0	0.62	0.039	0.65
Iron	+	0.10			trace						
Leucine					21.2	13.02					
Tyrosine					1.0	1.03					
Histidine					0.4	1.14	2.5*				
Arginine					2.7	1.06	14.5*				
Lysine					2.0	0.29	10.1*				
Ammonia							1.8*				
Humin-N.							I-6.8*				
							II-1.7*				

1. Gobley (25) From carp eggs.
2. Walter (26) From carp eggs.
3. Valenciennes and Frémy (8) From cartilaginous fishes.
4. Valenciennes and Frémy (8) From bony fishes.
5. Hugounenq (28) From the herring.
6. Galimard (29) Yolk protein of frog's eggs.
7. Steudel *et al.* (34) From herring roe.
8. Alsberg and Clark (30) From the eggs of the spiny dogfish.
9. Needham (31) From the eggs of the dogfish.
10. Levene (27) From cod-roe.

* Figures are for basic-N distribution. Non-basic-N = 61.8%. Additional figures include McClendon's (32) analysis of "Batrachiolin" from the eggs of Rana pipiens; N = 15.14%, S = 1.32%, P = 1.21%; and Fauré-Frémiet and Garrault (36) for carp ichthulin; N = 14.16%, P = 0.573% and trout ichthulin (37), N = 14.28%, P = 0.57%.

Riddle stated (45) in 1911, that these alternate layers are due to a daily rhythm of nutrition, probably associated with rhythmic changes in the blood pressure. The layering was quantitatively most significant in the last 5 to 8 days before extrusion, during which time more than 99 per cent of the yolk in the ovum was manufactured, months of slow growth being followed by a rapid increase in size after the diameter of 6 mm. had been reached. This sudden change might be due to an increase in permeability of the follicular cells. He was of the opinion that yolk formation, like the

majority of other types of tissue anabolism, was essentially reversible in nature, being in fact analogous to a reversible chemical change catalyzed by enzymes. He withheld opinion with regard to the production and possible reabsorption of the yolk *proteins*, but believed that fats were split before passing into the yolk. Arguing from the rapid growth in the later stages, he considered that in birds the yolk was not exclusively derived from the nucleus or from within the follicular cells. In Riddle's opinion the two chief factors in yolk formation were: (a) reversible action of enzymes, (b) partition coefficients of solubility in the yolk and in the maternal blood of the several yolk constituents. Spohr and Riddle (46) analysed the white and yellow yolk, and found that there was a very marked difference in composition. Whilst white yolk, formed at night, had a composition of water 86.7 per cent, phosphatides 1.13 per cent, neutral fat 2.39 per cent, extractives 0.40 per cent, ash 0.62 per cent, and protein 4.6 per cent, the yellow yolk, deposited in the day time had water 45.5 per cent, phosphatides 11.15 per cent, neutral fat 25.2 per cent, extractives 0.36 per cent, ash 0.44 per cent, and protein as high as 15.04 per cent. (Egg white contains 10–11 per cent of protein.) They pointed out that it is the *white* yolk which is at all stages in most intimate contact with the egg nucleus, the blastoderm, and the embryo. The percentage composition of the white yolk is nearer to that of living, growing tissues than is that of the yellow yolk.

Since the proportion of white yolk in the whole yolk of the hen's egg is probably less than 5 per cent (Riddle) the total contribution of the white yolk to the yolk proteins is small.

7. Iron and the Yolk Proteins—"Haematogen"

In the closed system of the egg, all the iron necessary for the synthesis of the haemoglobin in the blood of the embryo chick must obviously be present within the shell when the egg is laid. Actually, at the beginning of incubation, nearly all this iron is present in the form of an organic complex, not soluble in fat solvents, associated with the proteins of the yolk. From an average of 38 samples it has been found by us that the total yolk protein after completely extracting all the lipins with boiling alcohol contains 0.045 per cent of iron.

Peptic digestion of the yolk protein was found by Miescher (50) in 1870 to split off an insoluble product which Bunge (51) later named "haematogen." This material was investigated in more detail by Hugounenq and Morel (52, 53). The last authors found it to contain 0.455 per cent Fe and 8.7 per cent P, whilst Bunge had reported the smaller iron and phosphorus content of 0.29 per cent Fe and 5.19 per cent P. Hugounenq and Morel

(53, 54) believed that haematogen was a relatively complex substance, a type of conjugated protein in which an iron-containing pigment which they isolated and named "haematovin" was the prosthetic group, combined with a protein derivative. The combination was rather similar to that between globin and haematin to form haemoglobin. Haematovin, obtained as a brown powder by boiling haematogen with 30 per cent sulphuric acid, contained 2.6 per cent of iron and 0.1 per cent of phosphorus. It was insoluble in organic solvents but soluble in dilute alkali. Other than its content of organically combined iron, it had none of the characteristic properties of haematin, though certain oxidation products of haematin were obtained with an elementary composition similar to haematovin. Hugounenq and Morel regarded haematogen as a kind of undifferentiated, reserve haemoglobin and haematovin as "an incompletely differentiated and embryonic state of haematin." Some support for this belief lies in the more recently discovered fact that coincident with the sudden decrease in the vitellin of the yolk (Plimmer and Scott, 17) there is a corresponding rapid increase in the haemoglobin content of the chick about the 14th day (Sendju, 40).

Posternak (55) has recently prepared an enzyme-resistant, iron-containing material from the protein residue left after ether extraction of egg yolk. A fraction rich in iron (3.6%) and phosphorus (12.4%) was obtained after peptic and tryptic digestion of this residue.

It seems probable that haematogen is not a definite compound. It is likely that the iron-containing and the phosphorus-containing portions of the vitellin molecule are closely associated with one another, and are present in a portion of the molecule relatively resistant to enzyme hydrolysis. Depending on the extent to which the less stable portion of the protein molecule is hydrolyzed away, compounds containing more or less phosphorus and iron will be obtained in the "haematogen" separated. The ratio of Fe to P will probably be fairly constant (although the absolute values for the two elements will vary), provided the method of preparation is not dissimilar. This is exemplified by the analyses of Hugounenq and Morel given above (Fe:P = 1:19) compared with those of Bunge (Fe:P = 1:18).

Walter (56) prepared a compound from carp ichthulin in a similar way to that used by Bunge to prepare haematogen from vitellin. Walter's compound contained 0.25 per cent Fe and 2.6 per cent P.

Hugounenq and Morel (53), as would not be unexpected after Kossel's (11) demonstration that recognisable quantities of purines were not present in hen's eggs, found no xanthine bases in haematogen. Reducing sugar was also absent. It is worthy of mention here that, using the refined Krü-

ger-Schmid (97) technic, Mendel and Leavenworth (71) have since shown that a very small amount of purine N (0.0016 gm. N per egg) is present in fresh hens' eggs.

Effect of diet upon the Fe content of the egg. There was disagreement among earlier workers as to whether the iron content of egg yolk might be increased by feeding diets rich in iron. Hoffman (44) and Schmidt (43) claimed positive results, while Hartung (42) was unable to show such an effect. Recently Elvehjem, Kemmerer, Hart, and Halpin (41) state that the quantity of iron in egg yolk cannot be increased by feeding 50 mgm. Fe daily to the laying hen. (See also p. 98.)

Vitellinic acid. A compound with a similar Fe to P ratio was isolated by Levene and Alsberg (70) from vitellin by a method which did not entail an enzymic hydrolysis. Vitellin was suspended in water, and treated with strong ammonium hydroxide solution, which was allowed to remain in contact with the protein for two hours. The ammonia was then slowly neutralized with acetic acid, and excess of picric acid was added, and the reaction mixture filtered. On adding alcohol to the filtrate, a precipitate was obtained, purified as far as possible by repeated solution and reprecipitation, and finally extracted with boiling alcohol and ether. The product contained 0.57 per cent Fe and between 9.7 and 10.0 per cent P. Sulphur (0.3 per cent) was present. The Fe:P ratio (1:17 to 1:17.5) is quite close to that reported for haematogen. Levene and Alsberg's substance, which they call vitellinic acid, gave relatively large quantities of "melanin" on boiling with hydrochloric acid, and contained arginine and histidine. As might be expected from the relative lability of the phosphorus of phosphoproteins to alkali, this substance lost most of its phosphoric acid on warming with 2 per cent sodium carbonate. They showed that the iron was present in a relatively firm state of combination—i.e., organically bound.[2]

Assuming a 10 per cent P content for vitellinic acid, the maximum possible yield from vitellin (with a P content of about 1 per cent) is some 10 per cent of the weight of the vitellin. Kay and Marshall (95) in several experiments obtained yields of only 3.5 per cent or less of the weight of the vitellin. It would appear therefore that only a fraction of the vitellin phosphorus occurs in the vitellinic acid portion of the protein molecule. The approximate equivalent weight of vitellinic acid (2 specimens), using standard soda and thymol-phthalein, was found to be 203 and 216. It was found to contain, at the most, only traces of sulfur, apparently no tyrosine, but about 1 per cent of tryptophan (vitellin contains about 0.6 per cent

[2] Levene (27) prepared a very similar compound, "ichthulinic acid" from fish eggs.

of sulfur, 5 per cent of tyrosine, and 1.6 per cent of tryptophan (15)). Unlike the phosphopeptone derived from caseinogen, vitellinic acid appears not to lose its phosphoric acid on treatment with kidney phosphatase. It is therefore probably not a simple phosphoric ester.

The proteins of the hen's egg, like those of the milk in mammals, are secreted relatively rapidly by a comparatively small organ of the body. Ninety-nine per cent of the 3 grams or so of protein in the egg yolk is secreted during the last five to eight days before laying. Evidently one or both of two sources is possible for these proteins; they may be produced in the gland by the reversible action of proteolytic enzymes by direct combination of the amino acids circulating in the maternal blood plasma, or they may be derived (possibly by a process which does not entail complete breakdown to amino acid and resynthesis, but some far less drastic change in the protein molecule) from the circulating proteins of the blood plasma. Rimington (91) has pointed out that in their distribution of nitrogen as determined by Crowther and Raistrick (92), casein and lactoglobulin (serum globulin) are not remotely dissimilar, and has himself shown that artificially phosphorized serum globulin has several similarities to casein.

Whether or no any such considerations may be applied to the secretion of the egg-yolk proteins, i.e., whether they are synthesized mainly from the circulating amino acids of the mother's blood, or whether they are more or less simple modifications of the plasma proteins, we have as yet little more than surmise to guide us. It is perhaps unwise, even in surmise, to limit the possible alternative sources of the egg-yolk protein to the two just mentioned. In view of the high iron content of the egg-yolk proteins, and of the belief of some investigators that they contain an iron-rich nucleus which, with relatively few chemical changes is transformed directly into haemoglobin in the blood of the embryo chick (see p. 92 under haematogen), it is not impossible that the maternal red cells may be called upon for a contribution not merely of the iron, but of the iron attached to a fragment of fairly large molecular size, derived from haemoglobin by processes which involve only limited hydrolytic scission.

8. Phosphorus and the Egg-Yolk Proteins

A number of empirical facts have already been quoted with regard to the relationship of phosphorus to the egg-yolk proteins. Lecitho-vitellin, prepared in the usual way, contains organically combined phosphoric acid in two forms, one detachable from the protein molecule by boiling with ethyl and other alcohols, the other form more firmly united to the protein, and probably present in the iron-containing portion of the protein molecule.

Like the organically-bound phosphoric acid of casein, but unlike that of lecithin, vitellin phosphorus is readily liberated as inorganic phosphate by warming with alkali. Bayliss and Plimmer (72) suggested that the lecithin portion, detachable from lecitho-vitellin by alcohol, contains one-half of the total phosphorus of the protein. This finding is probably a fortuitous one, since the lecithin content of lecitho-vitellin varies widely with the method of preparation, and is probably far from a constant quantity. As already stated, Plimmer and Scott (17) showed that nuclein phosphorus was absent from vitellin. They later (73) give the figures shown in Table III for P distribution in the unincubated hen's egg.

TABLE III

PHOSPHORUS DISTRIBUTION IN UNINCUBATED EGGS. PERCENTAGE OF TOTAL PHOSPHORUS

	Hen's egg (Plimmer & Scott (73))	Frog's egg (Plimmer & Kaya (74))
(a) Inorganic P	trace	0
(b) Water-soluble organic P	6.2	4.3
(c) Ether-soluble P	64.8	26.2
(d) Phosphoprotein P	27.1	61.9
(e) Nuclein-like P	1.9	—
(f) Protein P (Phosphoprotein+nucleoprotein)	—	69.5

Unfortunately, in Plimmer and Scott's figures for the hen's egg, (e) is obtained by subtracting a+b+c+d from 100, so that its value as evidence of the presence of nucleoprotein or nucleic acid P is not outstanding. It would appear almost certain that any nucleic acid of fresh egg yolk is confined to the minute amount in the germ spot. Chapin and Powick (98) found that only 3.7 per cent of the total P of whole egg was present as inorganic phosphate. The remaining 96 per cent which is organically combined is almost entirely present in the yolk in the form of vitellin (some 16 per cent of the yolk) and phospholipins (about 11 per cent of the yolk). König (99) quotes Gobley as stating that 1.2 per cent of the yolk consists of glycerophosphoric acid, although Kay (100) found no substrate for phosphatase in fresh eggs. It is by no means difficult to obtain free glycerophosphoric acid, during the usual processes employed for precipitating proteins and lipins from the yolk, from its precursor the yolk lipin (100a), and the presence of this acid in the unincubated egg must be considered, at present, as being very doubtful. Pine (75) found the acid-soluble portion of the phosphorus of the egg to increase during storage.

Little is known with regard to the mode of linkage of the organically

combined phosphorus of lecithin-free vitellin with the rest of the molecule. It is, however, almost certainly present as an ester of phosphoric acid. Rimington (93) analyzed the phosphorus-rich peptone prepared by Rimington and Kay (94) from casein, and found that the phosphorus was associated with hydroxyamino acids, β-hydroxy-glutamic, hydroxyaminobutyric acids and probably serine being present in the peptone. It was concluded that the phosphoric acid was probably combined with hydroxy-groups of the hydroxy-amino acids. One-third of the phosphorus of the peptone appeared to be rather differently attached to the amino acids; the evidence suggested the possibility that in this fraction *two* of the hydroxyl groups of the phosphoric acid might be esterified. It is not unlikely that some similar form of combination is present in vitellin. In fact Posternak (76) has claimed the presence of a phosphorus-containing nucleus in vitellin, in which several serine-phosphoric acid complexes are combined.

As already noted, pure specimens of livetin contain very little phosphorus, but even after careful purification, 0.05 per cent P is so obstinately retained that it is probably a true component part of the protein molecule.

Egg yolk is similar to milk, another rapidly produced physiological secretion designed for the nutrition of the very young animal, in its high content of phosphoprotein and its very low nucleoprotein and inorganic phosphate content. In fact, outside these two sources, phosphoproteins hardly occur at all in nature. Like milk, egg yolk contains a fairly high proportion of fat, but it is dissimilar from milk in its high lecithin and iron, and very low carbohydrate content.

The fate of the phosphorus of the yolk during incubation was examined by Plimmer and Scott (73). Their results suggested that the phosphorus of the bone of the embryo chick came largely from the lipin of the yolk, whilst the vitellin P gave rise partly to additional inorganic phosphate and partly to the nucleoprotein of the tissues. As we do not purpose extending this review to cover the process of incubation, these interesting results will not be discussed here. It would, however, seem desirable that the work of Plimmer on the metabolism of phosphorus in the developing egg should be extended, using modern methods of phosphorus determination.

Analyses of the elementary composition of the egg and the yolk, and of the ash derived from these, are to be found in König's (99) book published in 1904. Some of these figures, which are to be found in several more recent text books, and are quoted as authoritative, are taken from Gobley's work (25a) published in 1846. Again it would seem high time that modern methods of analysis be applied to such an important dietary constituent as the egg.

9. Nutritional Value of Egg-Yolk Proteins

The proteins of the hen's egg must contain all the essential amino acids required for the growth of the young bird from an embryo of a few dozen cells to the complete chick ready for hatching. That is, the amino acids tryptophan, lysine, cystine, and histidine *inter alia* must be present in adequate quantities. It is in the highest degree likely that proteins biologically valuable for the growth of one species of vertebrate will be biologically valuable for another species, and this has been shown to be the case for egg proteins. Osborne and Mendel in 1912, 1913 (47) demonstrated that good growth is obtainable in rats with ovovitellin as the sole source of protein. Although their experiments were complicated somewhat by the fact that at that time very little was known with regard to the accessory food factors, their growth curves would suggest that vitellin was, in fact, superior for growth of rats to most of the proteins tested. De Sanctis (48) pointed out the value of egg yolk as a food for athreptic infants, and generally for infants who, though receiving the normal maximum diet, refused to grow. Smith and Chick (101) also bear testimony to the value of egg yolk in the diet of lactating rats whose litters were not thriving. This work was confirmed and extended by Clayton (48a) who found egg yolk superior to other food proteins for reproduction and lactation in the rat. In both these cases the situation is almost certainly controlled in the main by the vitamin content of the yolk, and probably hardly at all by the specific nutritional value of the egg-yolk proteins. To deal with the vitamin content of the egg yolk in this review would unfortunately lead us too far afield.

Mitchell and Carman (49) found in 1924 and 1926 that the protein of *whole* cooked egg had the highest biological value (94 per cent) of any they obtained from a food, surpassing even that of milk (85 per cent), the next highest, and of egg white (83 per cent). The relative figures for egg white and whole egg would suggest that the egg-yolk protein would be found to be even more valuable than that of the whole egg. Although we still lack adequate figures for the amino-acid composition of the egg-yolk proteins, certain at least of the biologically important amino acids (e.g., tryptophane and cystine) are known to be present in relatively large amounts in these proteins (see pp. 83 and 85).

10. The Effect of Diet on the Compositon of the Hen's Egg Yolk

That the composition of the egg yolk may be influenced by the diet of the fowl has been shown by Bethke, Kennard, and Sassaman (79) whose evidence indicated clearly that the vitamin A and vitamin D content of

egg yolk is largely, if not entirely, determined by the amount of these substances present in the diet. Whether the amino acid make-up of the protein is capable of influence in the same way was investigated by Pollard and Carr (77) and Gerber and Carr (78) and, from a different angle, by McFarlane, Fulmer, and Jukes (63). Pollard and Carr fed pigeons on diets restricted to a single seed grain for proteins and vitamins. On analysis, the resulting eggs showed a wide difference from one grain group to another in their total nitrogen. The distribution of nitrogen in six fractions also varied greatly in eggs from different diets. Only the eggs which gave a high melanin yield were hatchable. The formation of melanin is known to be dependent on the presence of tryptophan in the protein molecule. Gerber and Carr found that the mono-amino nitrogen was higher in the eggs obtained after the feeding of hemp, soy-bean or wheat, whilst the eggs obtained after feeding kafir showed a higher diamino-nitrogen content. They also obtained some evidence from anaphylactic reactions with guinea-pigs and rats that the egg proteins obtained from pigeons on the different diets differed immunologically. The rate of development of the embryo was also influenced markedly by the quality of the protein in the diet of the mother birds.

McFarlane, Fulmer, and Jukes took up the finding of Graham and Smith (96) that successful hatching of a fertile egg depended to a considerable extent on the nature of the protein supplement given to the hens in addition to the basal vegetable ration, and made a careful chemical investigation of the eggs produced by hens on different protein diets. They determined the amount of the essential amino acids tyrosine, tryptophane and cystine in both egg-white and mixed egg-yolk proteins, and found no significant difference in the content of any of these three amino acids in either the egg-white or the egg-yolk protein from one dietary group to another. In particular the percentage of tryptophan determined by three methods of analysis showed no significant variation. Nor did iron content of the yolk protein vary very materially from one group to another. There was, in fact, no clear evidence that the diet of the hen had any influence on the amino-acid or iron content of the egg-yolk (or egg-white) proteins.

These results are not, perhaps, surprising. The constancy of composition of the proteins of tissues and of secretion, from one individual in a species to another, whatever the diet, is a finding which has not yet been contradicted by any experimental evidence with the possible exception of that of Pollard and Carr just quoted. It is frankly difficult, however, to find direct experimental basis for the opinion, which would probably be held by most authorities on what appear to be à priori grounds, that any serious disturb-

ance in the content of essential amino acids in the diet of laying hens would lead first to a slackening of egg production, without any observable change in the composition of the individual proteins of the yolk (although their relative quantities might possibly vary), and would later result in complete cessation of egg production. It would seem that further work on this important aspect of nutrition not only with regard to egg protein, but with regard to other proteins resulting from anabolic and secretory processes, is urgently required, from the practical no less than from the more academic standpoint.

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